Departamento de Biología Molecular
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PROCESOS BIOCATALÍTICOS PARA LA PRODUCCIÓN DE CARBOHIDRATOS BIOACTIVOS: FRUCTOOLIGOSACÁRIDOS Y QUITOOLIGOSACÁRIDOS

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FRANCISCO JOSÉ PLOU GASCA, DR. EN CIENCIAS QUÍMICAS, INVESTIGADOR CIENTÍFICO DEL CSIC,

CERTIFICA: Que el presente trabajo "Procesos biocatalíticos para la producción de carbohidratos bioactivos: fructooligosacáridos y quitooligosacáridos", que constituye la Memoria que presenta la Licenciada en Biotecnología por la Universidad de Salamanca, Paloma Carmen Santos Moriano, para optar al grado de Doctora, ha sido realizado bajo su dirección en el Departamento de Biocatálisis del Instituto de Catálisis y Petroleoquímica del CSIC, Campus de Excelencia Internacional UAM + CSIC, Madrid.

Y para que conste, firma el presente certificado en Madrid en mayo de 2017,

Dr. Francisco J Plou

A mi abuelita Mariana

"*There is a light that never goes out*"
The Smiths

"Estallan los sentidos
en colores aún por inventar"
Toxicosmos, Los Planetas

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GENERAL INDEX

CONTENT INDEX

FIGURES INDEX

TABLES INDEX

ABBREVIATIONS AND ACRONYMS

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AC_{50}	half maximal antioxidant concentration
ANOVA	ANalysis Of VAriance
AOS	alginate oligosaccharides
B&R	Britton & Robinson
BAN-Glx	BAN immobilized on glyoxal agarose carriers
BET method	Brunauer-Emmett-Teller method
BHT method	Barrett-Joyner-Halenda method
BSA	Bovine Serum Albumin
Bt-chitosanase	chitosanase activity isolated from *B. thuringiensis*
CAZy	Carbohydrate-Active enZymes database
CBMSO	*Centro de Biología Molecular Severo Ochoa*
CHIT100	chitosan from Acros (100-300 kDa, >90% DD)
CHIT33	chitinase of 33 kDa from *Trichoderma harzianum*
CHIT42	chitinase of 42 kDa from *Trichoderma harzianum*
CHIT600	chitosan from Acros (600-800 kDa, >90% DD)
CIC BioGUNE	center for cooperative research in biosciences
CLEA	Cross-Linked Enzyme Aggregate
CLEC	Cross-Linked Enzyme Crystal
Co-A	coenzyme-A
COS	chitooligosaccharides
CSIC	*Consejo Superior de Investigaciones Científicas*
CSTR	Continuous Stirred Tank Reactor
d	dilution rate (h^{-1})
DALGEE	Dried ALGinate Entrapped Enzyme
DD	degree of deacetylation
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNS	3,5-dinitrosalicilyc acid
DP	Degree of Polymerization
DTT	dithiothreitol
DVS	divinyl sulfone
ELSD	Evaporative Light Scattering Detector
faCOS	N-acetylated chitooligosaccharides / chitin-oligosaccharides/fully acetylated COS
FAO	Food and Agriculture Organization of the United Nations
FBR	Fluidized-Bed Reactor
fdCOS	fully deacetylated COS
F_n	fructan with degree of polymerization *n*
FOS	fructooligosaccharides
GF_n	fructooligosaccharide with degree of polymerization *n*
GH	Glycoside Hydrolase family
GlcN	D-glucosamine
GlcNAc	N-acetyl-D-glucosamine
$(GlcN)_n$	chitooligosaccharide with degree of polymerization *n*
$(GlcNAc)_n$	chitin-oligosaccharide with degree of polymerization *n*
GOS	galactooligosaccharides
H	Hydrolysis activity
HILIC	hydrophilic interaction chromatography
HMO	Human Milk Oligosaccharide
HPAEC-PAD	High Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection
HPLC	High Performance Liquid Chromatography
iFBS	inactivated Fetal Bovine Serum
IMOS	isomaltooligosaccharides
IUB-IUPAC	International Union of Biochemistry - International Union of Pure and Applied Chemistry
K_M	Michaelis constant

LC-MS/MS	Liquid Chromatography- Mass Spectrometry/Mass Spectrometry
LEV	levansucrase
LEV-VS	levansucrase from *Z. mobilis* immobilized on vinyl sulfone-activated silica carriers
LMG	Low Molecular weight Glycan
LMW	Low Molecular Weight
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time Of Flight
ManNAc	N-acetyl-D-mannosamine
MBR	Membrane BioReactor
MS/ESI	Mass Spectrometry with Electrospray Ionization
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular Weight
NHE	Na^+/H^+ Exchangers
NMR	Nuclear Magnetic Resonance
PA	Pattern of Acetylation
paCOS	partially acetylated chitooligosaccharides
PBR	Packed-Bed Reactor
PDB	Protein Data Bank
2-PDS	2,2'-dithiodipyridine
PFR	Plug-Flow Reactor
QS1	chitosan from InFiQus (98 kDa, 81% DD)
ROS	Reactive Oxygen Species
rpm	revolutions per minute
SCFA	Short Chain Fatty Acids
SDS-PAGE	Sodium Dodecyl Sulphate – Poly-Acrylamide Gel Electrophoresis
SE	Standard Error of the mean
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy

SIdI	*Servicio Interdepartamental De Investigación*
SOS	soybean oligosaccharides
T	transfructosylating activity
T/H	Transfructosylation to Hydrolysis ratio
TEAC	Trolox Equivalent Antioxidant Capacity
TTC	2,3,5-triphenyltetrazolium chloride
U	units of activity
UAM	*Universidad Autónoma de Madrid*
USA	United States of America
v/v	volume/volume
V_{max}	maximum velocity
VS	vinyl sulfone
w/v	weight/volume
XOS	xylooligosaccharides

SUMMARY

Bioactive carbohydrates can have a positive impact in human health. This Thesis is focused in the enzymatic production, characterization and the study of the antioxidant properties of two families of bioactive oligosaccharides: fructooligosaccharides (FOS) and chitooligosaccharides (COS).

The production of FOS was carried out with the levansucrase from *Zymomonas mobilis.* This enzyme was able to produce from sucrose a mixture of levan-FOS, inulin-FOS and neo-FOS, all of them with prebiotic properties. The effect of reaction conditions (temperature and sucrose concentration) on product selectivity was studied. Levansucrase was covalently immobilized on vinyl sulfone-activated silica at neutral pH, rendering biocatalysts with notable apparent activity and operational stability.

Different strategies were addressed for the production of COS via enzymatic hydrolysis of chitosan. The COS mixtures were obtained using chitosans with different deacetylation degrees and a series of chitosanolytic enzymes: three commercial preparations (BAN, Neutrase and Rapidase), one enzyme extracted from the biopesticide *Bacillus thuringiensis* and two chitinases (CHIT33 and CHIT42) from *Trichoderma harzianum* expressed in *Pichia pastoris*. Enzymes were biochemically characterized and the reaction products were analyzed by a combination of chromatography and mass spectrometry. The chitosanolytic activity in BAN was covalently immobilized on glyoxal agarose carriers and a reactor for COS production was set up. To avoid the problems derived from the high viscosity of chitosan, a dual system was designed with a first step in a batch stirred tank reactor and a second step in a continuous packed bed reactor.

The antioxidant activity of two COS mixtures were analyzed: fully deacetylated COS (fdCOS) and partially acetylated COS (paCOS). Both mixtures showed scavenging effect on the ABTS radical cation. fdCOS were tested *in vitro* in neuroblastome cell cultures and showed statistically significant dose-dependent neuroprotective activity.

PRESENTACIÓN

Los carbohidratos bioactivos pueden tener un impacto positivo en la salud del ser humano. La presente Tesis Doctoral se centra en la producción enzimática, la caracterización y la actividad biológica de dos familias de oligosacáridos bioactivos: los fructooligosacáridos (FOS) y los quitooligosacáridos (COS).

Para la producción de FOS se utilizó la enzima levansacarasa de *Zymomonas mobilis*. Esta enzima, a partir de sacarosa, forma una mezcla de FOS de tipo levano, FOS de tipo inulina y neo-FOS, todos ellos con propiedades prebióticas. Se analizó el efecto de las condiciones de reacción (temperatura y concentración de sacarosa) sobre la selectividad de la reacción. La levansacarasa se inmovilizó de manera covalente en soportes de sílice activada con vinil sulfona a pH neutro obteniendo biocatalizadores con notable actividad aparente y estabilidad operacional.

En cuanto a la producción de COS, se llevaron a cabo diferentes estrategias para la hidrólisis enzimática de quitosano. Se obtuvieron varias mezclas de COS usando quitosanos con diferentes grados de desacetilación y una serie de enzimas quitosanolíticas: tres enzimas comerciales (BAN, Neutrase y Rapidase), una enzima extraída del biopesticida *Bacillus thuringiensis* y dos quitinasas (CHIT33 y CHIT42) de *Trichoderma harzianum* expresadas en *Pichia pastoris*. Se llevó a cabo la caracterización bioquímica de todas las enzimas así como el análisis de los productos de reacción mediante una combinación de cromatografía y espectrometría de masas. La actividad quitosanolítica de BAN se inmovilizó covalentemente en soportes de glioxal agarosa y se diseñó un biorreactor para la producción de COS. Para evitar los problemas derivados de la alta viscosidad del quitosano, se diseñó un sistema dual con una primera parte de hidrólisis parcial en un reactor agitado en *batch* y una segunda parte en un reactor de lecho fijo en continuo.

Por último, se analizó la actividad antioxidante de dos mezclas de COS: COS totalmente desacetilados (fdCOS) y COS parcialmente acetilados (paCOS). Ambas mezclas mostraron actividad neutralizadora del radical ABTS. La mezcla de fdCOS se analizó *in vitro* en cultivos celulares de neuroblastoma mostrando actividad neuroprotectora estadísticamente significativa y dependiente de la dosis.

1 INTRODUCTION & OBJECTIVES

1.1 Bioactive carbohydrates

Carbohydrates play an essential role in life. Composed basically of carbon, hydrogen and oxygen, they are the main source and store of energy for humans and most organisms. They are also long structural polysaccharides (like cellulose in plants or chitin in arthropods) and form biological molecules such as DNA, glycolipids, glycoproteins, etc. They are involved in cell recognition, cell adhesion and immunity, among other biological functions (Ramesh and Tharanathan, 2003). Carbohydrates can be randomly classified, according to their degree of polymerization (DP), in monosaccharides, oligosaccharides (2-10 units of monosaccharides) or polysaccharides (>10 units) as described by IUB-IUPAC terminology (IUPAC-IUB, 1982).

Apart from their intrinsic biological functions, some carbohydrates have other extra-nutritional beneficial effects on the organism, therefore receiving the name of bioactive carbohydrates (Aluko, 2012). Bioactive compounds, in general, are essential and non-essential molecules that occur in nature, part of the food chain, and have a positive effect on human health (Biesalski et al., 2009).

Table 1.1 and **Table 1.2** summarize some of the bioactivities of several carbohydrates from different sources, both polysaccharides (**Table 1.1**) and oligosaccharides (**Table 1.2**). It illustrates the variety of structures that carbohydrates can show: from short oligosaccharides such as lactulose, with prebiotic effect, to long chains of polysaccharides like chitosan, inulin or levan, with many bioactivities. Modified carbohydrates, such as sulfated sugars or uronic acids are also included in both tables.

This Thesis is focused in fructooligosaccharides (FOS) and chitooligosaccharides (COS), two kinds of oligosaccharides with different biological activities. Their enzymatic production and bioactivities are discussed in detail.

Table 1.1. Bioactivities of some polysaccharides and polysaccharide derivatives.

Type of carbohydrate	Name	Bioactivities	References
Aminoglycan	**Chitin**	Wound healing Antioxidant Antimicrobial	(Hamed et al., 2016)
	Chitosan	Antibacterial Antioxidant Antiviral Hypocholesterolemic Wound healing	(Foster et al., 2015) (Park et al., 2004) (Park and Kim, 2010) (Sugano et al., 1988) (Hamed et al., 2016)
Dextrin	**Enzyme-resistant dextrin**	Prebiotic	(Barczynska et al., 2012)
Fructan	**Inulin**	Prebiotic	(Sabater-Molina et al., 2009)
	Levan	Antidiabetic Anti-inflammatory Antioxidant Antitumor Antiviral Hypocholesterolemic Immuno-stimulatory Wound healing	(Srikanth et al., 2015)
Sulfated aminoglycan	**Dermatan sulfate**	Anti-inflammatory	(Belmiro et al., 2009)
	Heparin	Anticoagulant	(Luppi et al., 2005)
Sulfated sugar	**Fucoidan**	Anticoagulant Antidiabetic Antitumor Antiviral Antioxidant Hypocholesterolemic Anti-inflammatory Gastric protection Renal protection	(Shanthi et al., 2014) (Patankar et al., 1993) (Aisa et al., 2005) (Li et al., 2008)
	Naviculan	Antiviral	(Lee et al., 2006)
β-glucan	**Laminarin**	Immuno-stimulatory	(Kim et al., 2006)
	Lentinan	Anticoagulant Antioxidant Antiviral Antitumor	(Yang and Zhang, 2009) (Wang et al., 2013) (Yang and Zhang, 2009) (Ji and Yue, 2013)
	Oat β-glucan	Antidiabetic Hypocholesterolemic Antitumor Antimicrobial	(Daou and Zhang, 2012)
	Pachymaran	Antitumor	(Ramesh and Tharanathan, 2003)
	Schizophyllan	Antitumor	(Ramesh and Tharanathan, 2003)
	Scleroglucan	Antitumor	(Ramesh and Tharanathan, 2003)
	β-(1,3)-glucan (LMG)	Antioxidant	(Sun et al., 2009)

Table 1.2 Bioactivities of some oligosaccharides and oligosaccharide derivatives.

Type of carbohydrate	Name	Bioactivities	References
Aminoglycan	**Chitooligosaccharides (COS)**	Antibacterial	(Wu et al., 2013)
		Anticoagulant	(Kim and Rajapakse, 2005)
		Antihypertensive	(Park et al., 2003b)
		Anti-inflammatory	(Azuma et al., 2015) (Yoon et al., 2007)
		Antioxidant	(Chen et al., 2003) (Li et al., 2012)
		Antitumor	(Wu, 2012) (Azuma et al., 2015)
		Antiviral	(Artan et al., 2010)
		Immuno-stimulatory	(Azuma et al., 2015)
		Neuroprotective	(Jiang et al., 2014) (Huang et al., 2015) (Xu et al., 2010)
		Prebiotic	(Mateos-Aparicio et al., 2016)
Fructan	**Fructooligosaccharides (FOS)**	Prebiotic	(Sabater-Molina et al., 2009)
Galactan	**Galactooligosaccharides (GOS)**	Prebiotic	(Rodriguez-Colinas et al., 2013)
Hemicellulose	**Xylooligosaccharides (XOS)**	Prebiotic	(Vazquez et al., 2001)
	Arabino-XOS	Prebiotic	(Eeckhaut et al., 2008)
Starch	**Cyclodextrins**	Prebiotic	(Mussatto and Mancilha, 2007)
	Isomaltooligosaccharides (IMOS)	Prebiotic	(Kaneko et al., 1994)
	Isomaltulose	Prebiotic	(Mussatto and Mancilha, 2007)
	Gentiooligosaccharides	Prebiotic	(Mussatto and Mancilha, 2007)
Sulfated sugar	**Agar oligosaccharides**	Antioxidant	(Wang et al., 2004)
Synthetic sugars	**Lactulose**	Prebiotic	(Villamiel et al., 2002)
	Lactosucrose	Prebiotic	(Sako et al., 1999)
	Glycosylsucrose	Prebiotic	(Mussatto and Mancilha, 2007)
Raffinose	**Raffinose**	Prebiotic	(Mussatto and Mancilha, 2007)
	Soybean oligosaccharides (SOS)	Prebiotic	(Espinosa-Martos and Ruperez, 2006)
β-glucan	**Laminarin oligosaccharides**	Immuno-stimulatory	(Kim et al., 2006)
Uronic acid	**Alginate oligosaccharides (AOS)**	Antibacterial Antioxidant Prebiotic	(An et al., 2009) (Falkeborg et al., 2014) (Li et al., 2016)

1.2 Fructooligosaccharides

Fructooligosaccharides (FOS) are non-digestible carbohydrates composed of chains of fructose with different bonds with typically a terminal glucose linked by a α(1→2) bond. FOS are naturally present in many plants such as onion, asparagus, chicory, Jerusalem artichoke, leek, honey, tomatoes, etc. They are synthesized, processed and stored in cell vacuoles and serve as reserve carbohydrates (Roberfroid, 1993). FOS are also present in bacteria where they are part of extracellular polysaccharides having specific habitat-related functions (adhesion, defense, etc.) and occasionally serving as energy reserves (Sangeetha et al., 2005).

FOS can be divided in different types regarding the kind of linkage and structure (**Fig. 1.1**):

A. inulin-type FOS (^{1}F-FOS) with β(2→1) bonds between fructoses;
B. levan-type FOS (^{6}F-FOS) with β(2→6) bonds between fructoses;
C. neo-FOS (^{6}G-FOS) with a β(2→6) bond between glucose and fructose;
D. mixed FOS: highly branched fructans with both β(2→1) and β(2→6) linkages.

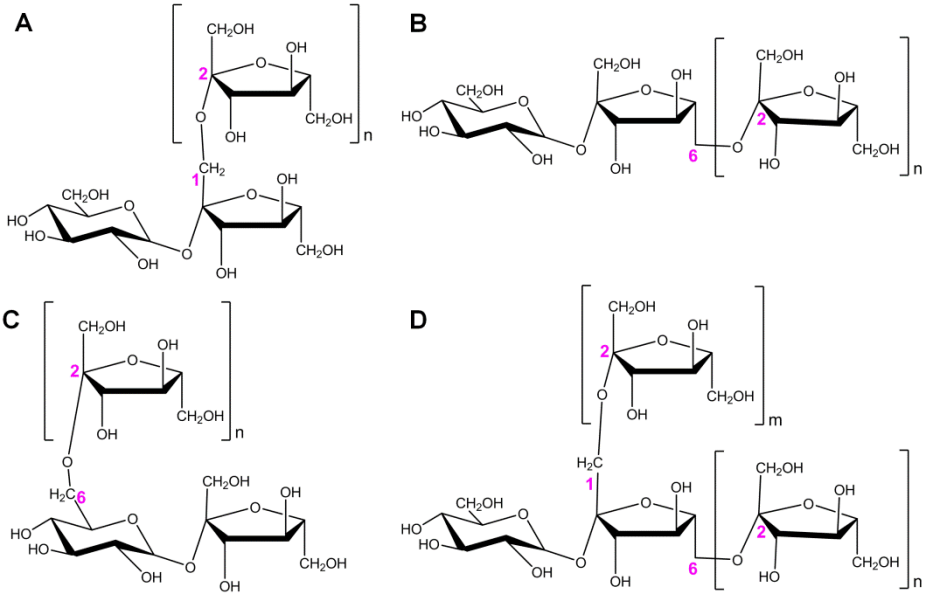


Fig. 1.1. Fructooligosaccharides structure. **A**. Inulin-type FOS. **B**. Levan-type FOS. **C**. Neo-FOS. **D**. Mixed FOS. n = 1-10; m = 1-10.

1.2.1 Inulin-type FOS

Inulin-type FOS are linear FOS with β(2→1) linkages. The first FOS of the inulin series is represented (1-kestose, when n=1) in **Fig. 1.1A**. They are the most widely studied kind of FOS and the more commercialized. **Table 1.3** summarizes the commercially available FOS for human diet supplementation, most of which are inulin-FOS. These FOS can be produced either by hydrolysis of inulin (oligofructose: GF_n and F_n with $n \leq 10$) or by transfructosylation of sucrose (GF_n with $2 \leq n \leq 10$).

Table 1.3. FOS commercially available for human consumption (adapted from Flores-Maltos et al., 2016, Corzo et al., 2015).

Production	Company & country	Commercial name	Structure	Degree of polymerization
Partial inulin hydrolysis	Cosucra (Belgium)	Fibrulose	GF_n & F_m β(2→1)	n= 1-6 m=2-7
	Beneo Orafti (Belgium)	Oligofructose Synergy1	GF_n & F_m β(2→1)	n= 1-6 m=2-7
	Jarrow Formulas (USA)	Inulin FOS	GF_n & F_m β(2→1)	n= 1-6 m=2-7
	Sensus (The Netherlands)	Frutalose	GF_n & F_m β(2→1)	n= 1-6 m=2-7
Sucrose transfructosylation	Beghin Meiji (Japan) Tereos (France)	Actilight	GF_n β(2→1)	n= 2-4
	GTC Nutrition Company (USA)	NutraFlora	GF_n β(2→1)	n= 2-4
	Meiji Seika Kaisha (Japan)	Meioligo	GF_n β(2→1)	n= 2-4
	Victory Biology Engineering Co. Ltd. (China)	Prebiovis scFOS	GF_n β(2→1)	n= 2-4
Agave hydrolysis	Nekutli (Mexico)	Metlos	GF_n & F_m β(2→6) β(2→1)	n.d.

n.d. not determined

1.2.2 Levan-type FOS

Levan is a β(2→6) linear polymer with minor β(2→1) branching, depending of the source. Levan can be found in plants (phlein) and microbes. They differ mainly in the MW of the polymer. Microbial levan is much larger [degree of

polymerization (DP) > 100; 2-100 million Da] and is produced as a reserve polysaccharide by a variety of microorganisms such as *Bacillus subtilis*, *Zymomonas mobilis*, *Aerobacter levanicum* and *Streptococcus salivarius* (Srikanth et al., 2015), among others. Plant levans are shorter (DP < 100) and, in general, highly branched (Guo, 2009, Monsan and Ouarne, 2009). Levan-type FOS are formed by β(2→6) linkages as schematized in **Fig. 1.1B** (6-kestose, when n=1). Unlike inulin-FOS, levan-FOS are not yet in the market despite their very interesting properties (Porras-Dominguez, 2014), mainly due to the difficulty of their production, which is described later in this chapter.

1.2.3 Neo-FOS

Neo-FOS (**Fig.1.1C**, neo-kestose when n=1) are fructooligosaccharides also with β(2→6) linkages but the first fructose is linked to the glucose moiety of sucrose, instead to the fructose like in the case of inulin– and levan-type FOS. Some studies suggest that this type of FOS possess enhanced properties compared with inulin-type FOS and better chemical stability (Gimeno-Perez et al., 2014, Kilian et al., 2002). Neokestose is found in various plants (Livingston et al., 1993) and as transfructosylation product of some microbial fructosyl-transferases (Bekers et al., 2002), but in small proportions.

1.2.4 Mixed FOS

Mixed fructans with β(2→6) and β(2→1) linkages are mainly found in plants. They can be branched inulins [with β(2→6) linkages at branch points] or branched levans [with β(2→1) linkages at branch points]. A representing short FOS of this kind is the molecule bifurcose (branched 6-kestose, **Fig. 1.1D** when n =1 and m = 1). Mixed FOS are present in the agave syrup. Agave is a genus of monocots native to the hot and arid regions of Mexico and the Southwestern United States. Agave stems are rich in branched fructans (>20% branching) of different DP and with β(2→6) and β(2→1) linkages. Its hydrolysis produces complex mixtures of FOS of different types but enriched in β(2→6) linkages (Praznik et al., 2013, Avila-Fernandez et al., 2011). These mixed FOS are commercialized by a Mexican company (Nekutli) under the name of Metlos® (**Table 1.3**).

1.2.5 Production of FOS

1.2.5.1 Inulin-FOS

In the case of inulin-type FOS, inulin is easily extracted from plants, generally chicory, and then hydrolyzed by inulinases (EC 3.2.1.7). Chicory roots contain about one fifth of their fresh weight in inulin (van Loo et al., 1995). For the transfructosylation of sucrose, sucrose fructosyltransferases (EC 2.4.1.99) and β-fructofuranosidases (EC 3.2.1.26) from fungi (generally *Aspergillus*) are typically employed. They catalyze the reaction of transfructosylation from sucrose in two steps (**Fig. 1.2**). First, forming a covalent fructosyl-enzyme intermediate in the catalytic site. Secondly, the intermediate is cleaved by the nucleophilic attack of the acceptor molecule. The acceptor can be another carbohydrate –forming the corresponding FOS– or a molecule of water –yielding the hydrolysis of sucrose-. The transfructosylation/hydrolysis ratio (T/H) depends on the enzyme, the substrate concentration and the reaction conditions.

1.2.5.2 Levan-FOS

For an industrial production of levan-type FOS, a cost-effective source of levan would be required for its hydrolysis by levanases (EC 3.2.1.65). Alternatively, enzymes that catalyze the transfructosylation of sucrose with formation of β(2→6) linkages could also be used (Bekers et al., 2002).

Enzymes that catalyze the formation of levan-type FOS and levan are called levansucrases (β-2,6-fructosyltransferases, EC 2.4.1.10). Levansucrases are bacterial extracellular enzymes that, according to the CAZy database (Henrissat and Davies, 2000), belong to the Glycoside Hydrolase (GH) family GH68. Levansucrases are found in microorganisms such as *Bacillus subtilis, Zymomonas mobilis, Microbacterium laevaniformans* or *Bacillus licheniformis* (Park et al., 2003a, Lyness and Doelle, 1983, Chambert et al., 1974). They have the same catalytic mechanism that the fructosyltransferases that form inulin-type FOS (**Fig. 1.2**). The type of bond formed between fructoses is reported to be mainly of the β(2→6) type, thus yielding 6-kestose and other FOS of the same family (**Fig. 1.1B**). However, production of other FOS such as 1-kestose (**Fig. 1.1A**), neokestose (**Fig. 1.1C**), and

blastose (Zambelli et al., 2014) has also been reported for some levansucrases. The ability of levansucrases to form β(2→1) linkages is not surprising considering the non-linear nature of levan, that contains some β(2→1) branching on the β(2→6) linear backbone. Some levansucrases also show levanase activity, yielding molecules of fructose, levanbiose, sucrose, levan oligomers or low molecular weight levan (Jang et al., 2003). Therefore, production of pure levan-FOS is quite difficult.

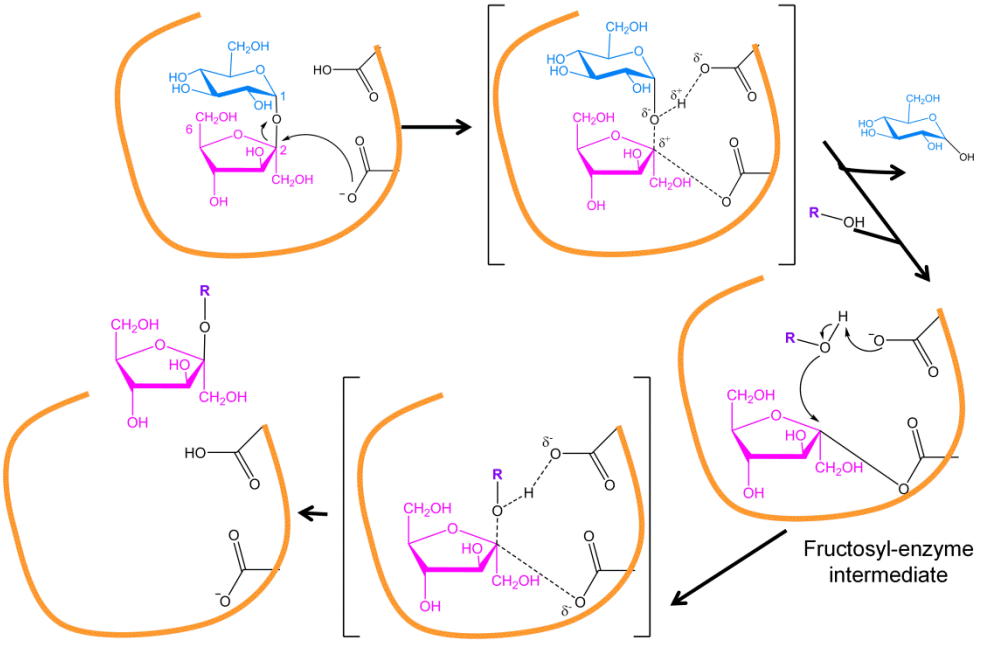


Fig. 1.2. Catalytic mechanism of a retaining β-fructofuranosidase or fructosyltransferase. R (purple) can stand for i) another molecule of sucrose or a FOS, yielding a FOS with DP n+1; ii) a molecule of water, yielding hydrolysis of sucrose into glucose and fructose.

Some levansucrases have been described and their biochemical characteristics studied (**Table 1.4**). They have been cloned, expressed and produced in heterologous hosts. However, their production in a cost-effective way and their application to industry has not yet been accomplished. Recently, Porras-Dominguez and cols. have proposed the production of FOS by hydrolysis of previously biosynthesized levan, mimicking the production of inulin-type FOS by hydrolysis of inulin (Porras-Dominguez et al., 2014). In this case, the products would be similar to oligofructose (GF_n and F_n) but linked by β(2→6) bonds.

Table 1.4. Levan and FOS biosynthesis by levansucrases from different microorganisms (adapted from Li et al. 2015).

Microorganism	Biocatalyst	Main products	References
Acetobacter diazotrophicus	Purified enzyme	1-kestose and levan	(Hernandez et al., 1995)
Acetobacter xylinum	Crude recombinant enzyme	Levan and FOS	(Tajima et al., 2000)
Bacillus amyloliquefaciens	Crude enzyme	Levan and FOS	(Tian et al., 2011)
	Purified recombinant enzyme	Levan	(Rairakhwada et al., 2010)
Bacillus licheniformis	Purified enzyme	Levan of different MW	(Nakapong et al., 2013)
	Purified recombinant enzyme	Levan of 9.6×10^6 Da	(Lu et al., 2014)
Bacillus megaterium	Purified recombinant enzyme	Levan and FOS	(Homann et al., 2007) (Strube et al., 2011)
Bacillus methylotrophicus	Crude enzyme	Levan MW 4-5 kDa	(Zhang et al., 2014)
Bacillus sp.	Crude enzyme	Levan of high MW	(Belghith et al., 2012)
	Purified enzyme	Levan of 6600 kDa and 6 kDa	(Ben Ammar et al., 2002)
Bacillus subtilis	Crude enzyme	Levan of 50-60 kDa	(Abdel-Fattah et al., 2005)
Geobacillus stearothermophilus	Purified enzyme	Levan and FOS	(Inthanavong et al., 2013)
Lactobacillus panis	Purified recombinant enzyme	Levan and 1-kestose	(Waldherr et al., 2008)
Lactobacillus reuteri	Purified enzyme	Levan of 150 kDa and 2000 kDa	(van Hijum et al., 2001)
	Purified recombinant enzyme	Levan	(van Hijum et al., 2004) (Biedendieck et al., 2007)
Lactobacillus sanfranciscensis	Purified recombinant enzyme	Levan > 5000 kDa	(Tieking et al., 2005)
Leuconostoc mesenteroides	Crude recombinant enzyme	Levan, 1-kestose, nystose, 1,1-kestopentaose and glucose	(Ishida et al., 2016)
	Purified recombinant enzyme	Levan, 1-kestose, nystose, 1,1-kestopentaose and glucose	(Kang et al., 2011, Kang et al., 2005)
Pseudomonas aurantiaca	Crude recombinant enzyme	Production of low-branched levan	(Jang et al., 2006)

Table 1.4. (cont.) Levan and FOS biosynthesis by levansucrases from different microorganisms (adapted from Li et al. 2015).

Microorganism	Biocatalyst	Main products	References
Pseudomonas chlororaphis subsp. *aurantiaca*	Purified recombinant enzyme	Levan and FOS	(Visnapuu et al., 2011)
Pseudomonas syringae pv. *phaseolicoa*	Purified enzyme	Levan 10^3-10^4 kDa	(Hettwer et al., 1995)
Pseudomonas syringae pv. *tomato*	Purified recombinant enzyme	Levan and FOS	(Visnapuu et al., 2011)
Zymomonas mobilis	Crude enzyme	Levan and FOS	(Bekers et al., 2002)
	Purified recombinant enzyme	Levan and hydrolysis products	(Sangiliyandi et al., 1999)

1.2.5.3 Neo-FOS

Neo-FOS are mainly produced by β-fructofuranosidases from yeasts such as *Xanthophyllomyces dendrorhous* (Linde et al., 2012, Gimeno-Perez et al., 2014) or fungi such as *Penicillium oxalicum* (Xu et al., 2015) or *Penicillium citrinum* (Lim et al., 2007). Neo-FOS are also found in small proportions as products of some levansucrases (Bekers et al., 2002).

1.2.6 Prebiotic properties of FOS

Human microbiota comprises $10^{12\text{-}14}$ microbial cells, which means about 1-1.5 kg of the total body weight (Clemente et al., 2012). It is not surprising that the gut microbiota has a substantial effect on health and disease in humans and it is sometimes referred as the "forgotten organ" (O'Hara and Shanahan, 2006). Gastrointestinal microorganisms are involved in energy harvest and storage, as well as in a variety of metabolic functions such as fermenting and absorbing undigested carbohydrates. More importantly, they are also closely related with the immune system.

FOS, as already stated in **Table 1.2,** are recognized prebiotics (Al-Sheraji et al., 2013). Prebiotics are described by the FAO like "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host well-being and health". The concept was first introduced by Gibson and Roberfroid in 1995, and

according to a later revision of the term (Roberfroid, 2007), there are certain requirements for an ingredient to be considered as prebiotic (Flores-Maltos et al., 2016):

- Low sensitivity to hydrolysis by saliva, pancreatic and intestinal enzymes or absorption along the gastrointestinal tract.
- To constitute a fermentable substrate for the intestinal microflora as established by scientific studies and to selectively stimulate growth and metabolism of one or more beneficial bacteria of the colon.
- To modify the composition of the colon flora, facilitating the development of beneficial species.
- To induce beneficial effects into the lumen or being systematically relevant for the health of the individuals.

FOS exhibit their prebiotic effect by two major mechanisms: by acting as trophic substrate of beneficial bacteria (*Lactobacillus* and *Bifidobacteria*, mainly) and therefore promoting their growth over pathogenic microorganisms; or by suffering fermentation into Short Chain Fatty Acids (SCFA). Also, some FOS can directly inhibit some pathogenic bacteria, or their fermentation can produce some bacteriocyn-like molecules that inhibit pathogenic bacteria. SCFA are acetate, butyrate and propionate in greater proportions, but also lactate, very important in infants, succinate and caproate. Production of SCFA leads to a series of effects, both local and systemic, that are schematized in **Fig. 1.3**. Local effects involve the reduction of the lumen pH. This acidification of the media enhances the growth of *Lactobacillus* and *Bifidobacteria* that, in terms, inhibits the growth of potentially pathogen microorganisms by competition. Systemic effects require the absorption of these SCFA by the colonic epithelial cells, which take place mainly by diffusion, but also with NHE (Na^+/H^+ exchangers) transporters that facilitate sodium and water absorption. Once inside the colonocytes, butyrate is an important source of energy for the cells. It can also have a direct action by inducing their proliferation and differentiation, and stimulating growth arrest and apoptosis of cancer cells by regulation of some genes. SCFA can be further incorporated to the blood torrent and reach the liver, where acetate and propionate can be transformed into acetyl-

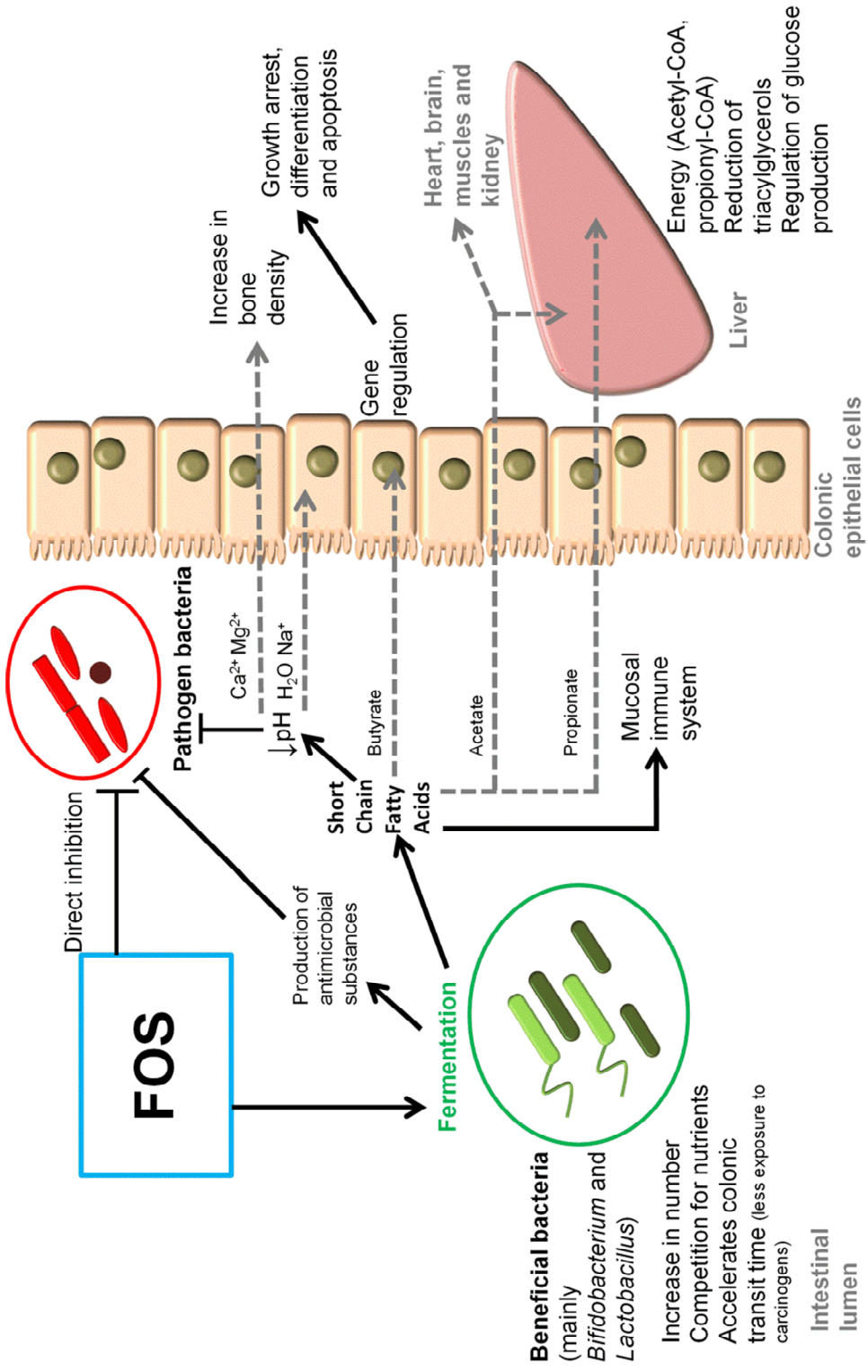


Fig. 1.3. Schematic representation of prebiotic mechanism of fructooligosaccharides.

CoA and propionyl-CoA respectively, molecules involved in the energy of the cells. From the liver, acetate and propionate can also regulate the glucose and triacylglycerol levels in blood (Roy et al., 2006). FOS can also initiate an anti-immune response in the colon, as a result of their changes in the microbiota (Capitan-Cañadas et al., 2016), enhancing local immune system.

In Europe there are only four types of non-digestible carbohydrates that are considered prebiotics with scientific evidence: FOS, GOS, lactulose and human milk oligosaccharides (HMOs) (Rastall and Gibson, 2015, Roberfroid et al., 2010), and the latter are not commercialized due to their complex structure and thus synthesis. FOS present in the market are, as described earlier in this Chapter, inulin-type FOS. Levan– and neo-FOS have been scarcely studied compared with inulin-type FOS. There are some studies which claim that these FOS with β(2→6) linkages (both levan– and neo-FOS) would exert an increased prebiotic activity and chemical stability, being a promising source of prebiotics (Marx et al., 2000, Kilian et al., 2002, Visnapuu et al., 2015).

Also, there are many other prebiotics being produced but that still lack strong scientific evidence. That is the case of XOS, COS, SOS, IMOS and the rest of oligosaccharides described as prebiotics in **Table 1.2,** which represent the so-called second generation prebiotics.

1.3 Chitooligosaccharides

Chitosan is a cationic polysaccharide composed of D-glucosamine (GlcN) and N-acetyl-glucosamine (GlcNAc) moieties with β(1→4) linkages that is obtained by partial deacetylation of chitin (poly-N-acetyl-glucosamine with β(1→4) linkages) (**Fig. 1.4**). Chitin is the second most abundant polysaccharide in nature after cellulose and, therefore, is an accessible and inexpensive source for the production of bioactive molecules. Chitin is usually found in nature as 90% acetylated (Kim and Rajapakse, 2005).

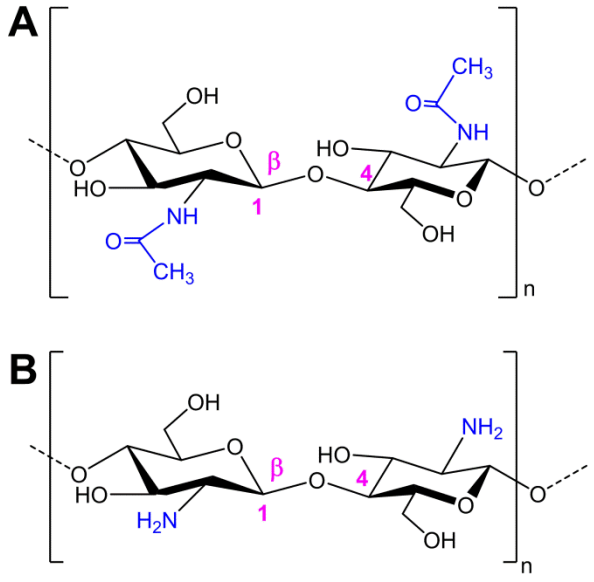


Fig. 1.4. Chemical structures of chitin (**A**) and fully deacetylated chitosan (**B**).

Chitin is widely distributed in nature in the exoskeletons of arthropods such as crustaceans (lobster, crab, shimp or krill) and insects (fly, cockroach, ladybird or silkworm), the cell walls of fungi (like *Aspergillus niger* or *Penicillium notatum*), the radulae of mollusks (squid, cuttlefish or octopus), the scales and other soft tissues of fish and lissamphibians, and in brachiopods, cnidarian and siboglinidae (Hamed et al., 2016). Recently, endogenous presence of chitin in vertebrates has also been described (Tang et al., 2015).

Every year, more than 10^{10}-10^{11} tons of chitin are produced by living organisms, from which more than 10,000 tons could be available from shellfish waste (Hamed et al., 2016). Therefore, re-valorization of chitin for the production of biological active molecules has arisen quite a big interest in recent years, considering that there is a current lack of acceptable waste management options for this kind of by-products.

As described before, partial deacetylation of chitin leads to chitosan, the only natural cationic polysaccharide known. Chitosan and chitin both have very interesting biological properties (**Table 1.1**) but their applications are limited by their viscosity and poor solubility at neutral pH. On the other hand, they are biocompatible, biodegradable and non-toxic (Aranaz et al., 2014). Chitosan is widely used as a biopolymer in the pharmaceutical and food industries as a carrier for drugs or as a preservative because of its antibacterial properties. Also, it has many applications in the cosmetic, dermatological, textile, agriculture and paper industries (Zou et al., 2016). Chitin and chitosan are also widely used as solid supports for enzyme immobilization (Krajewska, 2004).

Chitosan biological activities depend on its molecular weight (related to the polymerization degree, DP), its charge (related to the deacetylation degree, DD), and the pattern of acetylation (PA).

1.3.1 Production of chitooligosaccharides

Chitooligosaccharides (COS), obtained by hydrolysis of chitosan, are water soluble and have many biological activities (**Table 1.2**). They are also significantly less viscous than chitin and chitosan. Chitosan can be hydrolyzed for the production of COS by physical, chemical or enzymatic methods. Whereas the two first require extreme reaction conditions and the composition of the final product is difficult to control (Yang and Yu, 2014), the use of enzymes involves milder conditions (moderate temperature, neutral or slightly acidic pH, atmospheric pressure, etc.) and a better reproducibility of the process.

Regarding enzymatic hydrolysis, DP and DD of the products depend, apart from the source of chitosan used as substrate, on the specificity of the enzyme employed as biocatalyst. Chitosanolysis can be catalyzed mainly by chitosanases (EC 3.2.1.132) but also by other enzymes including exo-β-glucosaminidases (EC 3.2.1.165), exo-β-N-acetylglucosaminidases (EC 3.2.1.30), chitinases (EC 3.2.1.14) and, non-specifically, by proteases (Li et al., 2007), pectinases (Kittur et al., 2003a), cellulases (Xia et al., 2008) and lysozyme (Lin et al., 2009, Pantaleone et al., 1992).

Chitosanases would specifically endo-hydrolyze chitosan through GlcN-GlcN linkages. However, there are some subclasses that could also hydrolyze GlcNAc-GlcN and GlcN-GlcNAc linkages. Chitinases hydrolyze specifically GlcNAc-GlcNAc bonds. As described before, chitosan is usually not 100% deacetylated, and that would explain the activity of chitinases on some chitosans (Thadathil and Velappan, 2014). Exo-β-glucosaminidases and exo-β-N-acetylglucosaminidases release the monomers from the non-reducing end of chitosan/chitin yielding GlcN and GlcNAc units as final products, respectively (**Fig. 1.5**).

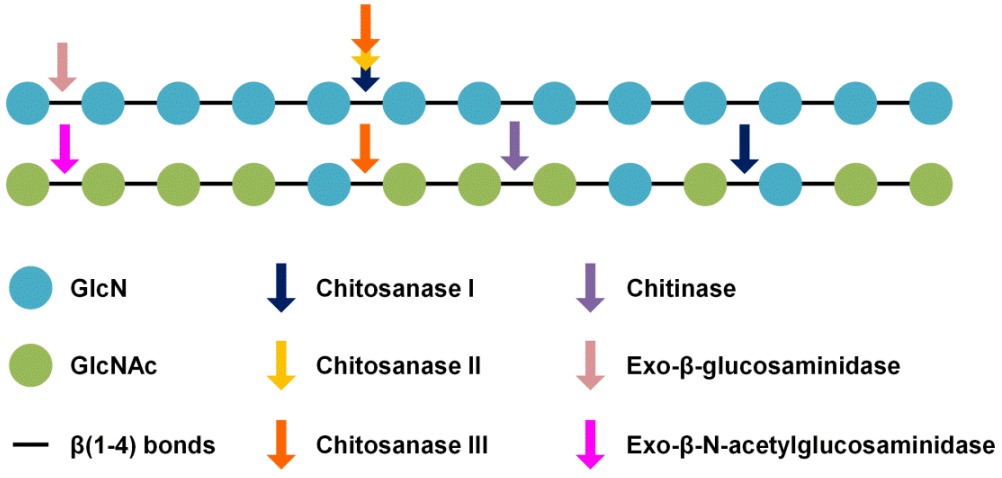


Fig. 1.5. Schematic representation of the acting sites of the different enzymes that hydrolyze chitosan and chitin.

Chitosanases belong to families GH5, GH8, GH46, GH72 and GH80, according to the CAZy database (Hoell et al., 2010). GH46 chitosanases, produced mainly by *Bacillus* and *Streptomyces*, are the ones that are better known in terms of

structure and catalytic mechanism. Also, chitosanases from GH72 family are widely studied, and are mainly of fungal origin (*Aspergillus*, *Trichoderma*, etc.).

Hydrolysis of chitosan by unspecific enzymes was first described by Pantaleone in 1992 (Pantaleone et al., 1992). This statement strongly contradicts one of the principles of enzymology, which is specificity. However, promiscuity of some enzymes is known, and in the case of cellulases, pectinases and lysozyme the natural substrates are quite similar to chitosan in terms of structure and bonds (**Fig. 1.6** and compared with **Fig. 1.4**) (Aiba, 1992, Kittur et al., 2003a, Xia et al., 2008). However, in other studies that describe chitosanolysis with some commercial enzymes of very different nature (lipases, proteases, amylases, etc) that are not completely pure, it is not possible to discard the presence of another contaminant enzyme in the preparation that would be responsible for this activity (Montilla et al., 2013, Li et al., 2007, Lee et al., 2008). Chitosanolysis with the use of commercial enzymes has the advantage of its availability and the approval for their use in some specific industries like food or pharmaceutical.

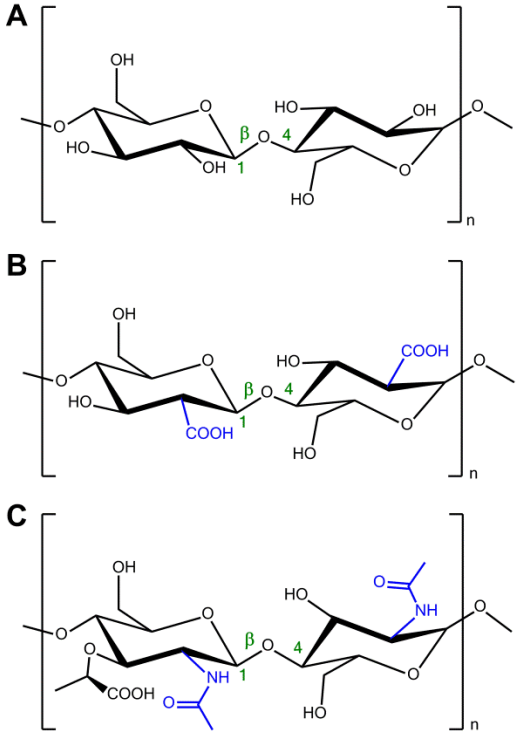


Fig. 1.6. Chemical structures of cellulose (**A**), pectin (**B**) and peptidoglycan (**C**).

A great challenge in the production of COS is the identification and separation of COS mixtures. A complex mixture of fully deacetylated COS (fdCOS) and partially acetylated COS (paCOS, with variable acetylation pattern) is commonly obtained. In addition, the low availability of COS standards, especially of paCOS, complicates the analysis. Methods to separate COS include size exclusion chromatography (SEC) (Song et al., 2014), hydrophilic interaction liquid chromatography (HILIC) and anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Xiong et al., 2009, van Munster et al., 2015, Mekasha et al., 2016). COS are poorly retained at alkaline pH on anion-exchange columns typically employed to separate carbohydrates, thus requiring unusual mobile phase conditions. Mass spectrometry (MS) techniques, such as LC-MS/MS (Kim et al., 2013) or MALDI-TOF (Montilla et al., 2013), are also of great potential, especially combined with NMR spectroscopy (Mahata et al., 2014).

1.3.2 Biological activities of chitooligosaccharides

COS have enhanced biological activities compared with the polymers chitin and chitosan. They are reported to show antimicrobial, antioxidant, antiviral, antiangiogenic, antitumoral, anti-inflammatory and prebiotic properties, as reported in **Table 1.2**. More in detail, **Table 1.5** shows some of the recent studies on COS and chitosan activities. Some of the most important bioactivities and their mechanisms are described below:

- **Antibacterial** activity of chitosan seems to be stronger than the activity of the oligomers. The mechanisms by which chitosan and COS perform their bacterial growth inhibition is due to the presence of the amino group at C-2 position that is positively charged. This positive charge interacts with the negatively charged carboxylic acid group of the macromolecules of bacterial cell surface. The formation of these complexes would block the permeation of nutrients to the cell. This explains why the more charges (higher DP and DD), the more inhibition (Kim and Rajapakse, 2005). This antimicrobial activity shows great potential for the use of chitosan and COS as food preservatives or functional food additives (Xia et al., 2011).

Table 1.5. Some examples of chitosan and chitooligosaccharides bioactivity assays.

Biological activity tested	COS tested	System tested	Relevant results	References
Antibacterial	Chitosan and COS (Different MW)	Gram (+) and Gram (-) bacteria	Chitosans showed more antibacterial activity than COS Chitosan showed stronger bactericidal activity towards Gram (+) bacteria	(Xia et al., 2011)
Anti-inflammatory	COS mixture MW 1.2 – 5.3 kDa DD 80-85%	*In vivo* paw edema model	Sowed the anti-inflammatory effects in a dose and MW dependent manner	(Fernandes et al., 2010)
Antioxidant	COS mixture $(GlcN)_{2-5}$	*In vitro* chemical assays	The mixture had scavenging activity of 50% compared with ascorbic acid	(Sinha et al., 2012)
Antitumor	Mixture of $(GlcN)_4$ $(GlcN)_5$	*In vivo* mice model	Inhibition of S180 tumor cell growth by oral and intraperitoneal administration	(Jeon and Kim, 2002)
Immunostimulatory	paCOS mixture DP 3-10	Fish model	Activation of phagocytic activity in macrophages	(Liu et al., 2014)
	$(GlcN)_{1-6}$ $(GlcNAc)_{1-6}$ Chitin Chitosan	*In vitro* macrophages model	Oligomers enhanced migratory activity of macrophages whereas polymers reduced it	(Okamoto et al., 2003)
Prebiotic	COS with different DD and MW	*In vitro* fermentations	faCOS might decrease some human microbiota populations COS could increase *Lactobacillus/Enterococcus*	(Mateos-Aparicio et al., 2016)

- **Antioxidant** activity of COS can be measured by their ability to scavenge different free radicals. Several studies show that COS with lower DP have stronger antioxidant activity, being glucosamine and chitobiose the most active (Chen et al., 2003, Li et al., 2012, Mengibar et al., 2013). Regarding DD, a study with different chitosans showed that the most deacetylated chitosans had the highest radical scavenging effects on various radicals (Park et al., 2004). Mengibar and cols. also established that mixtures with a high percentage of acetylated residues could be considered non-antioxidant (Mengibar et al., 2013). However, the relationship between DD and the pattern of acetylation (PA) with antioxidant activity might not be so clear, since

Li and cols. demonstrated that partially acetylated chitotriose had more antioxidant activity than fully deacetylated chitotriose, showing that the acetylation played an important role in the antioxidant activity of COS (Li et al., 2013).

- **Antiviral, antitumor and anti-cancer** activities of COS might all be related with the **immuno-stimulatory** effect. The stimulation of leucocytes, cytotoxic T-cells and natural killer cells would inhibit tumor growth (Kim and Rajapakse, 2005, Azuma et al., 2015). Antitumor activity is also related with the cytotoxicity of COS. Glucosamine is highly and unspecificly cytotoxic. This effect can be antagonized by combination with COS (de Assis et al., 2012). Also, there are some studies that show the inhibition of angiogenesis by some COS mixtures (Wu et al., 2012, Xiong et al., 2009), pointing chitohexaose as the most active COS.
- COS also show **anti-inflammatory** activity (Azuma et al., 2015). In general, COS are able to inhibit the production of pro-inflammatory cytokines in a DP and dose dependent manner. This anti-inflammatory activity can also enhance the antitumor activity described above. All the COS studied present a high deacetylation degree. However, a dependency on DD with anti-inflammatory activity has not yet been analyzed.
- **Prebiotic** activity of COS has recently been studied *in vitro* with batch fermentations, showing significant changes over growth of *Lactobacillus* and *Enterococcus* (Mateos-Aparicio et al., 2016). However, more studies *in vivo* and in humans are needed for COS to be considered as prebiotics.

These activities, like in the case of chitosan, depend mostly on their DP and DD, which, in turn, depend on the origin of chitosan and the hydrolysis method. It is worth noting that most of the studies are performed with fully acetylated (faCOS) or fully deacetylated COS (fdCOS), or with mixtures of partially acetylated COS (paCOS) where only the DD is determined, but not the exact structure of the COS, related with their PA.

1.4 Immobilization of enzymes

Immobilization of enzymes is a useful strategy that might favor the industrial development of processes for the production of bioactive carbohydrates. In 1971, at the first Enzyme Engineering Conference, Katchalski-Katzir defined immobilized enzymes as "enzymes physically confined or localized in a certain defined region of space with retention to their catalytic activities, and which can be used repeatedly and continuously" (Katchalski-Katzir and Kraemer, 2000). Most immobilization processes involve the conversion of a water-soluble enzyme into a solid form of a catalyst, which facilitates separation, reutilization of the catalyst, and downstream processes. Immobilization often implies an enhancement of enzyme stability towards pH, temperature or organic solvents. Although immobilization of enzymes has been studied in the last decades, there are no established rules to be applied for each enzyme, and the selection of an immobilization method remains almost empirical (Torres-Salas et al., 2011). The selection of a proper support and the optimization of the immobilization conditions (pH, buffer, temperature, enzyme/support ratio) are always cumbersome (Fernandez-Arrojo et al., 2015).

1.4.1 Immobilization methods

There are three major components of an immobilization system: the enzyme, the carrier and the mode of attachment. In general, the methods can be divided in three main groups, represented in **Fig. 1.7**: binding to a carrier, crosslinking, and entrapment (Sheldon, 2007). In **Fig. 1.7** the enzyme is represented with a pink protein and the carrier like a light blue sphere. However, this does not mean that all carriers are spherical, and although it is represented on the surface of the carrier, some supports are porous and the immobilization takes place also on their inside.

- **Immobilization on carriers**

It involves the attachment of the enzyme to the surface of a solid carrier or support. Enzyme supports can be classified in organic and inorganic (Brena et al., 2013). Among the organic supports, they can be natural polymers, such as polysaccharides (cellulose, agarose, chitosan, etc.), or synthetic polymers, such as polystyrene, polyacrylamide, smart polymers (poly-N-isopropylacrylamide),

polymethacrylate, etc. Inorganic supports can be divided into natural minerals (silica, bentonite, alumina, etc.) and processed materials (glass, protein-coated microcrystals or metals). Binding to the carrier can take place in a covalent or non-covalent fashion, depending on the kind of interactions formed between the enzyme and the support.

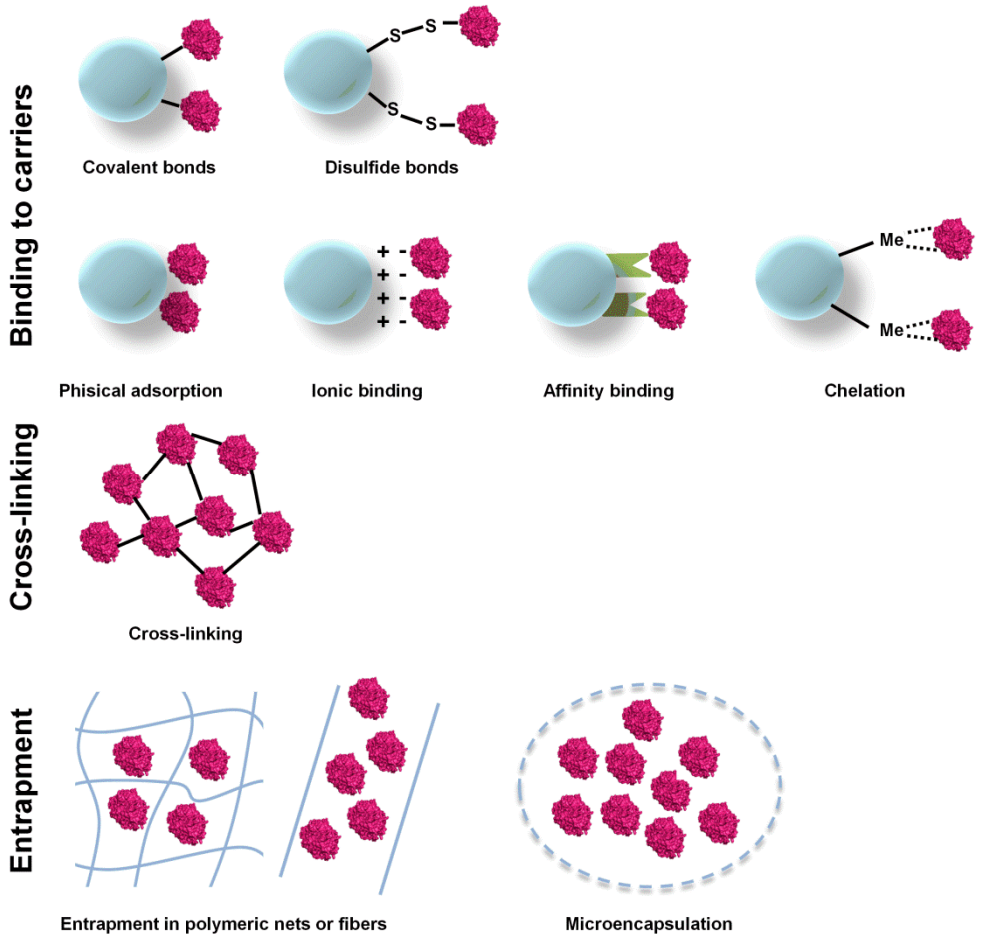


Fig. 1.7. Schematic representation of the most representative immobilization methods.

Immobilization by **non-covalent binding** to carriers includes physical adsorption, where the enzyme binds to a non-functionalized carrier by unspecific forces such as dipole-dipole, hydrogen bonds, hydrophobic or Van der Waals forces; ionic binding to charged carriers; affinity binding, for example with the use of antibodies; or by chelation, when the carrier is functionalized with a transition metal that binds to different parts of a protein, e.g. a His-tag. Physical and ionic adsorption are the

most straightforward kinds of immobilization, but they usually present the disadvantage of the easily desorption of the enzyme from the carrier at industrial conditions (Fernandez-Arrojo et al., 2015).

Immobilization by **covalent binding** is based on the formation of covalent bonds between enzyme and carrier. It gives rise to a tight union so that the enzyme does not separate during utilization. In general, the covalent bonds involve the amine (lysines), thiol (cysteines), carboxylic (glutamic and aspartic acids) and/or phenoxyl (tyrosines) residues of the protein. Different materials, e.g. polysaccharides (agarose), organic polymers (polymethacrylate) or controlled-porous silica can be chemically activated with reactive groups (glyoxal, epoxy, hydroxy-succinimide, ethyl dimethylaminopropyl carbodiimide, etc.) to covalently attach enzymes (Torres-Salas et al., 2011). Formation of disulfide bonds is a special case of reversible covalent binding through a free cysteine on the surface of the enzyme (Ovsejevi et al., 2013).

- Cross-linking

This strategy combines both covalent binding and entrapment. It is based on the formation of covalent bonds between enzyme molecules without the use of a carrier. It requires the use of bifunctional reagents such as glutaraldehyde. There is an increasing interest in carrier-free enzymes such as cross-linked enzyme crystals (CLECs) (St. Clair and Navia, 1992) and cross-linked enzyme aggregates (CLEAs) (Sheldon et al., 2005), because they offer highly concentrated enzyme activity in the catalyst and high stability. Although CLECs are quite expensive, immobilization by cross-linking usually translates in a reduction in the production cost owing to the exclusion of an often expensive carrier (Sheldon and van Pelt, 2013).

- Entrapment

This method of immobilization involves the confinement of the enzyme within a polymeric matrix. There are different approaches like gel or fiber entrapment and micro-encapsulation in semipermeable membranes. One interesting entrapment approach is the use of calcium alginate and its further dehydration to form Dried ALGinate Entrapped Enzymes or DALGEEs (Fernandez-Arrojo et al., 2013).

There are some cases where various methods are combined. For example, cross-linking can be applied together with almost every method. An enzyme can be cross-linked before immobilization by entrapment to increase the size of the enzyme and avoid its leakage through the matrix pores (Wu et al., 2006); or after covalent immobilization to enhance the stability of the biocatalyst (Lopez-Gallego et al., 2005). Another interesting approach is the so-called "smart immobilization" that involves the use of magnetic nanoparticles for the rapid removal of the biocatalyst from the reaction media (Sheldon and van Pelt, 2013).

1.4.2 Production systems with immobilized biocatalysts

Most bioprocesses are of no significance if they cannot be applied on industrial scale. A reactor that operates with a biocatalyst is called a bioreactor. Bioreactors can operate in batch or continuous mode. Immobilized enzymes allow the production of FOS and COS in a continuous way, which enhances the overall productivity of the system. Some of the most common type of bioreactors employing immobilized biocatalysts are listed below (Eş et al., 2015) and represented in **Fig. 1.8**.

- **Batch reactor:** it consists of a tank vessel with a stirrer where the substrate and the biocatalyst are mixed and kept under the reaction conditions until the biotransformation is complete. Then, ideally, only the products are removed while the biocatalyst is kept in the vessel, where it is cleaned and ready to be used in another cycle of reaction (Fernandez-Arrojo et al., 2015).
- **Continuous stirred tank reactor (CSTR)**: it is a tank vessel with a stirrer that can be a turbine or a propeller that is continuously fed with the substrate and the products are removed. It is very simple and can be applied to a variety of systems. It is an ideal-flow reactor, meaning that composition of the reaction mixture is the same at every point of the reactor because they assure good mixing. Main drawbacks would be low productivity and mechanical damage of biocatalyst caused by agitation (Rakmai and Cheirsilp, 2016).
- **Packed bed reactor (PBR)**: PBRs are formed by immobilized enzymes packed into a column where the substrate is continuously fed through the inlet and the product is recovered at the outlet. Productivity is quite high compared

with CSTRs (Rakmai and Cheirsilp, 2016). However it is not easily applied to systems with heterogeneous or very viscous substrates because of the large pressure drop during operation (Patel and Majumder, 2011, Rodriguez-Colinas et al., 2016). In PBRs reactants are assumed to move through the reactor without mixing at a constant velocity. Concentration rate varies along the column and can be related with reaction time.

- **Fluidized bed reactors (FBR)**: FBRs are column reactors in which the column is not completely packed. There is a porous plate at the bottom by which a gas passes upwards and, as velocity of the gas increases, the bed becomes fluidized. The main advantage of this bioreactor is the excellent heat transfer (Lorenzoni et al., 2015).

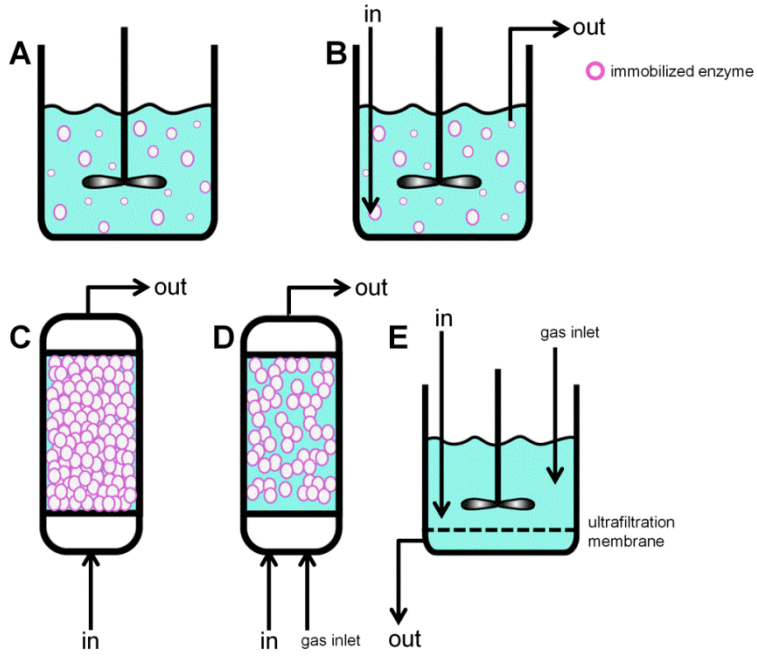


Fig. 1.8. Schematic representation of some of the most common reactor types. **A**. Batch reactor. **B**. Continuous stirred tank reactor. **C**. Packed bed reactor. **D**. Fluidized bed reactor. **E**. Membrane reactor.

Membrane bioreactors (**Fig. 1.8E**) are a special case of reactors where the enzyme is retained within a micro– or nano–filtration membrane and can be recycled without the need of being previously immobilized. The substrate is fed and the products are continuously removed in the permeate (Kuroiwa et al., 2009, Sinha et al., 2014).

1.5 Objectives

The main objectives of this Thesis are: (1) the development of enzymatic processes for the production of fructooligosaccharides (FOS) and chitooligosaccharides (COS); (2) the development of analytical methods for the analysis of complex reaction mixtures containing FOS and COS; and (3) the study of the biological properties of synthesized FOS and COS.

To achieve these goals, several specific objectives were proposed:

- Biotransformation of sucrose into FOS with the use of levansucrase.
- Immobilization of levansucrase for the optimization of bioprocesses for the production of FOS.
- Screening of chitosanolytic activity in commercial preparations, non-conventional yeast cultures or other sources.
- Enzymatic hydrolysis of chitosan into bioactive COS using chitosanolytic enzymes.
- Optimization of analytical methods (chromatography and mass spectrometry) for the identification of reaction products.
- Immobilization of chitosanolytic enzymes and set-up of bioreactors for the production of COS.
- Comparative analysis of the antioxidant and neuroprotective activity of synthesized FOS and COS.

2 MATERIALS & METHODS

2.1 Materials

2.1.1 Enzymes

Levansucrase (LEV) from *Zymomonas mobilis* was obtained as a powder preparation from Amano Enzyme Inc. (Nagoya, Japan). Rapidase TF and Klerzyme 150 were kindly donated by DSM (Heerlen, NL). Pectinex Ultra SP-L, Neutrase 0.8L, BAN 480L, NovoShape, Ultraflo L, Shearzyme 2X, Pentopan Mono Conc. BG, Flavourzyme and Alcalase were gracefully donated by Novozymes (Bagsvaerd, Denmark). β-Glucanase from *Bacillus amyloliquefaciens* (E-CELBA) was acquired from Megazyme (Wicklow, Ireland). *Bacillus thuringiensis* var. *aizawai* (XenTari®) was purchased from A-Z Drying (Osage, IA, USA). Transglutaminase was purchased to Ajinomoto (Tokio, Japan) as Activa WM.

Chitinases CHIT33 and CHIT42 from *Thricoderma harzianum* were produced and kindly supplied by the laboratory of Dr. Maria Fernández Lobato (*Centro de Biología Molecular Severo Ochoa*, CBMSO, UAM-CSIC).

2.1.2 Reagents

Sucrose, glucose and fructose were from Merck (Darmstadt, Germany). 1-Kestose and nystose were from TCI Europe (Zwijndrecht, Belgium). 1F-Fructosylnystose was from Megazyme (Wicklow, Ireland). Chitosans were either acquired from Acros Organics (CHIT100 and CHIT600) or produced by InFiQuS (QS1) (Madrid, Spain) (**Table 2.1**). COS standard (MW ≤ 2000, DD ≥ 90%) was purchased from Qingdao BZ Oligo Biotech Co. Ltd. (China). Glycol chitosan, D-glucosamine (GlcN), N-acetyl-glucosamine (GlcNAc), and levan from *Zymomonas mobilis* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fully deacetylated COS with DP from 2 to 5, and fully acetylated COS with DP from 2 to 4 were purchased from Carbosynth Ltd. (Berkshire, UK). 6-Kestose, blastose, neokestose and neonystose were purified in the laboratory as previously described (Alvaro-Benito et al., 2007, Linde et al., 2012, Zambelli et al., 2014). Sodium alginate was from FMC BioPolymer (Philadelphia, PA, USA).

All other reagents were of the highest purity grade.

Table 2.1 Chitosans used in this Thesis.

Chitosan	MW	DD	Supplier
QS1	98 kDa	81%	InFiQus
CHIT100	100-300 kDa	≥ 90%	Acros Organics
CHIT600	600-800 kDa	≥ 90%	Acros Organics

2.1.3 Immobilization carriers

Vinyl sulfone-activated silica carriers were prepared as described elsewhere (Morales-Sanfrutos et al., 2010) and kindly supplied by the laboratory of Dr. Francisco Santoyo Gonzalez (*Departamento de Química Orgánica, Universidad de Granada*, Spain).

High Density Glyoxal 4BCL (spherical, 50-150 μm diameter, 40-60 $\mu mol_{glyoxyl}$ mL_{gel}^{-1}, coupling capacity 15-20 mg_{BSA} mL_{gel}^{-1}), was purchased from Agarose Bead Technologies (Miami, FL, USA).

2.1.4 Buffer solutions

The buffer solutions employed in this Thesis are described in **Table 2.2**.

Table 2.2 Buffer solutions used in this Thesis.

Buffer	Salt	Concentration	pH
Levansucrase reaction and stability buffer	Sodium acetate	50 mM	5.4
Neutrase, Rapidase, BAN and *Bacillus thuringiensis* chitosanase reaction buffer	Sodium acetate	100 mM	5.0
Chitinases CHIT42/CHIT33 reaction buffer	Sodium acetate	100 mM	5.5
VS-activated silica immobilization buffer	Potassium phosphate	10 mM	7.0
Glyoxal-agarose immobilization buffer	Sodium bicarbonate	10 mM	10.0
Britton & Robinson buffer	Acetate-phosphate-borate	100 mM	2.0-11.0
Buffer for COS production at larger scale	Ammonium acetate	100 mM	5.0

2.2 Activity assays

2.2.1 Levansucrase

The activity of levansucrase was determined using 100 g/L sucrose in 50 mM sodium acetate buffer (pH 5.4) as substrate following the release of reducing sugars in 20 min by the 3,5-dinitrosalicylic acid (DNS) assay adapted to 96-well plates (Ghazi et al., 2007). One unit of activity (U) was defined as that corresponding to the release of one µmol of reducing sugar per minute. Calibration curves were made with glucose.

2.2.2 Chitosanolytic enzymes

Chitosanolytic activity was also determined by the DNS method with slight modifications. Prior to the assay, low-molecular-weight contaminants in the commercial enzymes were removed with a DP-10 desalting column (GE Healthcare, Uppsala, Sweden). Activity assays were performed in 1.5 mL centrifuge tubes by adding 200 µL of enzyme to 800 µL of 1% (w/v) chitosan CHIT100 dissolved in 50 mM sodium acetate buffer (pH 5.0). Tubes were incubated at 50°C and 900 rpm in a Thermo Shaker TS-100 (Boeco, Hamburg, Germany) and reactions were stopped by addition of 0.25 M NaOH in a 1/1 (v/v) ratio. The addition of NaOH also caused the precipitation of the remaining polysaccharide, which was removed by centrifugation at 5,000 x g for 10 min. Reducing sugars were detected in the supernatant. One unit of activity (U) was defined as that corresponding to the release of one µmol of reducing sugar per minute. Calibration curves were made with glucosamine.

2.3 Biochemical characterization of enzymes

2.3.1 Protein concentration

Protein concentration was determined by the Bradford assay (Bradford, 1976) adapted to 96-well plates. Bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO, USA) was used as standard for the calibration curve.

2.3.2 SDS-PAGE

Proteins were visualized by electrophoresis in denaturing conditions in 12% acrylamide gels (Laemmli, 1970). Samples were prepared as follows: 15 µL of enzyme preparation conveniently diluted was mixed with 5 µL of 4 x loading buffer with β-mercaptoethanol and heated for 10 min at 96°C. Gel was stained with ProtoStain Blue (National Diagnosis, Atlanta, GA, USA) and bands were compared with molecular weight markers (Precision Plus Protein™ All Blue Prestained Protein Standards, BioRad, USA).

2.3.3 Zymograms

Levansucrase activity in gel was assayed with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) reagent as described by Mukasa and cols. (Mukasa et al., 1982). After running the polyacrilamide gel (12%) in native conditions, it was washed twice with 50 mM sodium acetate pH 5.6 containing 0.5% (v/v) Triton X-100. Then it was incubated for 30 min with 1 M sucrose in sodium acetate 50 mM pH 5.6 and room temperature. After washing the gel with water three times, it was stained with 1% (w/v) TTC in 0.25 M NaOH at 100ºC. Reaction was stopped with 5% (v/v) acetic acid.

Chitosanolytic activity in gel was assayed by native polyacrylamide gel electrophoresis in 12% gels without SDS containing 0.1% (w/v) glycol chitosan following the Laemmli method (Laemmli, 1970). After electrophoresis the gel was soaked in 100 mM sodium acetate buffer (pH 5.0) with 1% (v/v) Triton X-100 and incubated for 2 h at 37°C. The gel was washed with distilled water and stained with 0.1% (w/v) Congo red. The contrast was enhanced for the development of dark blue color with the addition of 5% (v/v) acetic acid. Chitosanase activity was observed as a clear area against a dark blue background.

2.3.4 Optimal temperature and pH

For the determination of optimal temperature of the enzymes, 90 µL of substrate in the reaction buffer and 10 µL of enzyme were incubated at different temperatures in a Thermo Shaker TS-100 (Boeco, Hamburg, Germany) for a fixed time and then stopped by addition of alkali. The concentration of reducing sugars was then measured by the DNS assay. For the determination of optimal pH, 100 µL solutions containing 10 µL of enzyme and 90 µL of substrate in B&R buffer (Britton and Robinson, 1931) at different pH values (2.0-9.0) were incubated at the optimal temperature in a Thermo Shaker TS-100 (Boeco, Hamburg, Germany) for a fixed time. After stopping the reaction, the concentration of reducing sugars was measured by the DNS assay. The relative activity was referred to the activity of the enzyme at the optimal conditions of temperature and pH, respectively.

2.3.5 Kinetic constants

Different reactions were set with the same amount of enzyme and different concentrations of substrate. Activity was measured by the standard DNS assay as described in Chapter 2.2.1, under the optimal reaction conditions. Graphs were represented with SigmaPlot software and curves were fitted to a Michaelis-Menten hyperbole. Kinetic constants (V_{max} and K_M) were extracted from the hyperbole formula.

2.3.6 Enzyme stability

Stability towards pH was assessed incubating the enzyme extract at different pH values (2.0-11.0) in B&R buffer (Britton and Robinson, 1931) at room temperature, and thermostability was analysed incubating the enzyme preparation in the stability buffer at different temperatures (30-75°C). Reactions were incubated, aliquots were taken at different times, and the residual activity (referred to the activity of the enzyme prior to any incubation) was measured by the DNS assay under the standard conditions.

2.3.7 Statistical analysis

All the experiments were performed in triplicate and results are expressed as the mean ± standard deviation.

2.4 Analytical methods

2.4.1 SEC-HPLC

Size exclusion chromatography (SEC) was performed using a ternary pump (Varian) coupled to a PolySep-GFC-P 4000 column (300 x 7.8 mm) from Phenomenex (Terrace, CA, USA) kept at 25ºC by the MFE-01 oven (Analisis Vinicos, Tomelloso, Spain). Samples were automatically injected by Hitachi L-2200 autosampler (Hitachi, Japan). Mobile phase was H_2O at 0.6 mL/min for levan and 0.25 M ammonium acetate (pH 4.7) for chitosan. Signal was detected by an evaporative light scattering detector (ELSD 2000ES, Alltech, Lexington, KY, USA). ELSD conditions in both cases were set at 115ºC and a nitrogen flow of 3.5 L/min.

2.4.2 HPAEC-PAD

The identification of oligosaccharides was carried out by High Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) at 30°C on a Dionex ICS3000 system (Dionex, Thermo Fischer Scientific Inc., Waltham, MA, USA) consisting of an SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, and an autosampler (model AS-HV). All eluents were degassed by flushing with helium. Depending on the reaction mixtures three different methods were used that are described in Table 2.2. The chromatograms were analyzed using Chromeleon software (Dionex, Thermo Fischer Scientific Inc., Waltham, MA, USA). The identification and quantification of the different carbohydrates was done on the basis of commercially available standards. Mobile phase compositions are detailed in **Table 2.3**.

Although all the methods have been developed for this thesis, HPAEC-PAD is a quite common technique in the case of FOS (Rodriguez-Gomez et al., 2015, Zambelli et al., 2014). On the other hand, the method for COS identification by this chromatographic technique is quite a novelty. Therefore, it is thoroughly explained below.

Table 2.3. HPAEC-PAD methods for FOS and COS analysis. A: 200 mM NaOH. B: H_2O. C: 200 mM CH_3COONa. D: 100 mM NaOH, 320 mM CH_3COONa.

		Method					
Carbohydrate	Column	Time (min)	A	B	C	D	Flow rate (mL/min)
FOS	CarboPack PA1 (4 x 250 mm) + CarboPack PA1 guard (4 × 50 mm)	0	10	90	0	0	1
		13	10	90	0	0	1
		20	50	30	20	0	1
		30	50	30	20	0	1
		35	50	0	50	0	1
		37	50	0	50	0	1
Longer FOS and inulin	CarboPack PA200 (4 x 250 mm) + CarboPack PA200 guard (4 × 50 mm)	0	50	50	0	0	0.5
		15	50	0	0	50	0.5
		60	0	0	0	100	0.5
		95	0	0	0	100	0.5
COS	CarboPack PA200 (4 x 250 mm) + CarboPack PA200 guard (4 × 50 mm)	0	2	98	0	0	0.3
		30	2	98	0	0	0.3
		50	50	0	50	0	0.5

In the case of COS, with the aid of commercial standards, GlcN (peak *1*), $(GlcN)_2$ (*2*), $(GlcN)_3$ (*3*), $(GlcN)_4$ (*4*), $(GlcN)_5$ (*5*), GlcNAc (*6*), $(GlcNAc)_2$ (*7*), $(GlcNAc)_3$ (*8*), and $(GlcNAc)_4$ (*9*) were identified by HPAEC-PAD (**Fig. 2.1**) and further quantified by building calibration curves. In general, with PA200 columns, the order of elution typically correlates with the increasing DP because more sugar moieties imply a higher negative charge to interact with the positive stationary phase. However, it is noteworthy that the retention time in both series (acetylated and deacetylated) did not follow such order –e.g. $(GlcN)_2$ eluted later than $(GlcN)_3$–, probably due to the unusual eluting conditions (4 mM NaOH) that do not allow complete ionization of carbohydrates. This fact represented an extra difficulty for the elucidation of the unknown peaks, further increased by the low availability of paCOS standards. Also, longer COS would co-elute with GlcN.

An additional problem is that alkaline mobile phases typically employed in HPAEC-PAD methods may cause epimerization of the N-acetyl-D-glucosamine moiety to N-acetyl-D-mannosamine (ManNAc) (Lee, 1996), thus artificially increasing the number of substances in the sample. We observed that the low

concentration of NaOH (4 mM) employed in our system did not promote epimerization of GlcNAc into ManNAc, at least during the time of analysis (30 min).

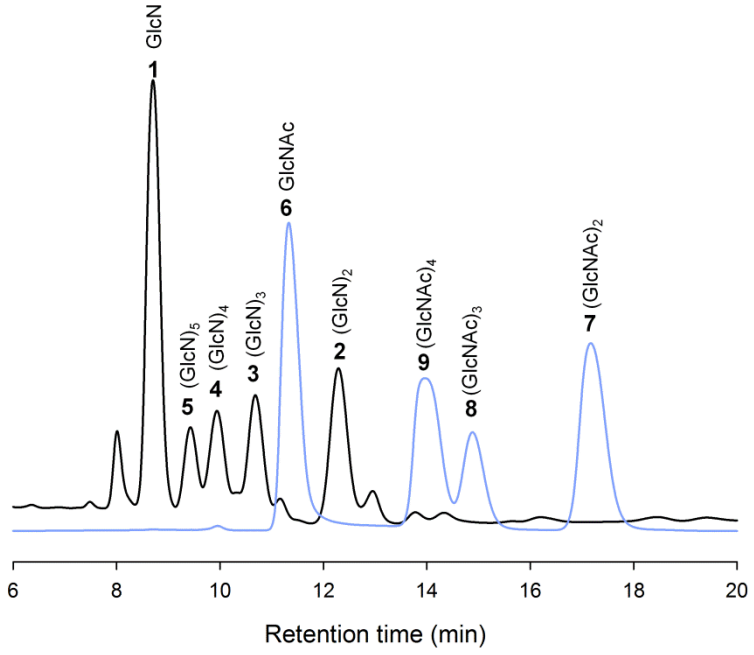


Fig. 2.1. Separation of COS by HPAEC-PAD. Method "COS" of Table 2.3. Black chromatogram: deacetylated series (fdCOS). Blue chromatogram: acetylated series (faCOS).

2.4.3 Semipreparative HPLC

Some unknown peaks from the levansucrase reaction were purified using semipreparative HPLC with a Delta 600 pump (Waters, Milford, MA, USA) and a Kromasil-NH_2 column (5 µm, 10 x 250 mm, Analisis Vinicos, Tomelloso, Spain). A three-way flow splitter (model Accurate, Dionex, Thermo Fischer Scientific Inc., Waltham, MA, USA) and a refraction index detector (model 2410, Waters, Milford, MA, USA) equilibrated at 30ºC were used. Acetonitrile/water 68:32 (v/v), degassed with helium, was used as mobile phase at 4.7 mL/min. The column temperature was kept constant at 30ºC.

2.4.4 Mass spectrometry

Mass spectrometry analyses of unknown carbohydrates were made in the *Servicio Interdepartamental de Investigación* (SIdI, UAM).

For MS/ESI assays the equipment was a mass spectrometer with hybrid QTOF analyzer (model QSTAR, Pulsar i, AB Sciex, Framingham, MA, USA). Reaction samples were analyzed by direct infusion and ionized by electrospray (with methanol as ionizing phase) in positive reflector mode.

For MALDI experiments, the equipment was a Ultraflex III TOF/TOF (Bruker, Billerica, MA, USA) with a NdYAG laser. Registers were taken in positive reflector mode within the mass interval 40-5000 Da, with external calibration and with 20 mg/mL 2,5-dihydroxybenzoic acid in acetonitrile (3:7) (v/v) as matrix. Samples were mixed with the matrix in a 4:1 proportion and 0.5 µL were analysed.

2.4.5 Nuclear magnetic resonance (NMR)

The structure of the purified carbohydrates was elucidated by NMR in collaboration with Dr. Jesús Jiménez Barbero and Dr. Ana Poveda (CIC Biogune, Basque Country, Spain). A combination of ^{1}H and ^{13}C-NMR, 1D and 2D (COSY, NOESY, HSQC, HSQC-TOCSY, HMBC) techniques was employed. The spectra of the samples (ca. 10 mM), dissolved in deuterated water, were recorded on a Bruker (Billerica, MA, USA) AVANCE DRX500 spectrometer equipped with a tuneable broadband 1H/X probe with a gradient in the Z axis, at a temperature of 298 K. Chemical shifts were expressed in ppm with respect to the 0 ppm point of DSS, used as internal standard. All the experiments were run using standard sequences provided by Bruker. COSY and NOESY (500 ms mixing time) experiments were performed with 8 and 64 scans, respectively, with 384 increments in the indirect dimension and with 1024 points in the acquisition dimension. The spectral widths were 4 ppm in both dimensions. The HSQC, HSQC-TOCSY (60 ms mixing time) and HMBC experiments were performed with 8, 16 and 64 scans, respectively, with 384 increments in the indirect dimension and with 1024 points in the acquisition dimension. The spectral width was 70 ppm (80 ppm for the HMBC) in the indirect dimension (^{13}C) and 3 ppm in the acquisition one (^{1}H). All the 2D experiments were transformed to a 4096 x 4096 matrix in order to improve resolution and resolve the overlapping signals as much as possible.

2.5 Production of FOS and levan by levansucrase

2.5.1 Production of levan

Production of levan by levansucrase was studied under different conditions and followed by size exclusion chromatography (SEC). Reactions with 5 U/mL levansucrase were incubated in an orbital shaker (Vortemp™, LabNet, Edison, NJ, USA) using 100 or 600 g/L sucrose in 50 mM acetate buffer (pH 5.0) at 4ºC or 40ºC. Aliquots were taken at 0 h, 2 h, 24 h and 48 h, inactivated with 0.5 volumes of 0.4 M Na_2CO_3, filtered with 0.45 µm nylon syringe filters (Análisis Vínicos, Tomelloso, Spain) and diluted 1:5 and 1:10 with H_2O before SEC analysis. Molecular weight of the products was estimated by comparison with dextran standards (180-140,000 Da, Phenomenex, Torrance, CA, USA).

2.5.2 Production of FOS

To a solution of 600 g/L sucrose in 50 mM sodium acetate buffer (pH 5.4) levansucrase (5 U/mL) was added and the mixture incubated at 40ºC with gentle stirring. Aliquots were taken at different times, inactivated with 0.5 volumes of 0.4 M Na_2CO_3, filtrated with 0.45 µm nylon syringe filters and conveniently diluted before analysis. The identification of fructooligosaccharides was carried out by HPAEC-PAD as described in **Table 2.2**. The identification of higher oligosaccharides was done by comparison with an inulin standard (Raftiline, Orafti, Belgium).

2.5.3 Purification of oligosaccharides

Unknown fructooligosaccharides were purified using semipreparative HPLC as described earlier. After collecting the different oligosaccharides, the mobile phase was eliminated by rotary evaporation in an R-210 rotavapor (Büchi, Germany). Isolated products were characterized by mass spectroscopy and NMR.

2.6 Production of COS

2.6.1 Rheology of chitosan solutions

Viscosity of chitosan CHIT100 in 100 mM sodium acetate buffer pH 5.0 was studied. Experiments were performed with a rheometer (Advanced rheometer AR20, TA Instruments, New Castle, DE, USA) and a conic geometry (6 cm, 1º) for small volume samples (1 mL), and with standard size recessed end concentric cylinder geometry for larger samples (10 mL). Results were analyzed with TAData32 software. Viscosity of reaction samples was analyzed without precipitation with NaOH. Instead, the enzyme was inactivated by heating at 95ºC for 10 min. Considering the fact that chitosan is a non-Newtonian fluid, shear force *vs.* shear stress flow charts were adjusted to the best fit (Herschel-Bulkley model) and the effective viscosity was calculated.

2.6.2 Analysis of chitosan hydrolysis by SEC-ELSD

To 960 µL of a 1% (w/v) chitosan solution in 50 mM sodium acetate buffer (pH 5.0), the enzyme (40 µL) was added. Reactions were incubated at 50°C in 1.5 mL centrifuge tubes at 900 rpm with orbital stirring. At different times, aliquots were taken, diluted with water, and filtered with 0.45 µm cellulose filters (Analisis Vinicos, Tomelloso, Spain). Samples were analyzed by SEC as described above.

2.6.3 Characterization and quantification of COS by HPAEC-PAD

Reactions were performed as previously described for the SEC-ELSD experiments with one difference. In this case, aliquots were mixed with 0.25 M NaOH in a 1/1 (v/v) ratio to stop the reaction and to precipitate the remaining polysaccharide, which was removed by centrifugation at 5,000 x g for 10 min. The COS fraction was diluted with water (2.5 mM NaOH final concentration) and analyzed by HPAEC-PAD as described in **Table 2.3**.

2.6.4 Extraction and purification of Bacillus thuringiensis chitosanolytic activity

For extraction of the chitosanolytic activity from the *B. thuringiensis* preparation, 2 g cells were mixed with 25 mL of distilled water, vortexed and centrifuged for 10 min at 5,000 x g. This step was repeated three times to remove soluble

contaminants of the preparation. Then, 25 mL of 1 M sodium acetate were added to the pellet, vortexed and incubated at 40°C for 1 h in a rocking incubator (Enviro-Genie®, Scientific Industries Inc., Bohemia, NY, USA). Cells were centrifuged at 5,000 x g for 20 min. Pellet was discarded and the supernatant was filtrated with 0.45 µm cellulose acetate/nitrate filters. After filtration, the sample was concentrated 10-fold with an Amicon® Ultra-50 system (10 kDa ultrafiltration membrane, Merck Millipore, Billerica, MA, USA) to eliminate the excess of salts, diluted 10-fold with 50 mM sodium acetate buffer pH 5.0, and concentrated again with the same 10 kDa membrane followed by Amicon® Ultra-15 tubes (3 kDa membrane, Merck Millipore, Billerica, MA, USA). Protein concentration was determined by the Bradford method (Bradford, 1976). Activity was assayed by the standard DNS method.

2.6.5 Large-scale production and purification of COS

A reaction for the production of deacetylated COS was set with Neutrase as biocatalyst and 2% (w/v) CHIT600 in 100 mM ammonium acetate buffer pH 5.0 as substrate. Reaction for the production of partially acetylated COS was set with CHIT42 as biocatalyst and 2% (w/v) chitosan QS1 as substrate. After 48 h at 50ºC and 900 rpm, the enzyme was separated from the reaction by ultrafiltration with Amicon® system (10 kDa membrane) or Amicon Ultra-15 (3 kDa membrane). After separation, the COS were lyophilized and further dried in a desiccator with phosphorous pentoxide. Final COS mixture was analyzed by HPAEC-PAD and mass spectrometry.

2.7 Enzyme immobilization

2.7.1 Characterization of carriers

Porosity: Porosity of carries was determined in the "*Unidad de Apoyo*" of ICP-CSIC. Nitrogen isotherms were performed at the temperature of liquid N_2 (–196°C), using a Micromeritics ASAP 2420 device. The samples were previously degassed at 80°C for 16 h to a residual vacuum of 5·10-3 torr, to remove any loosely-held adsorbed species. The surface area of the support was determined by the BET method (Brunauer et al., 1938). The distribution of pore sizes was established from the adsorption branch of the isotherm using the BJH model (Barrett et al., 1951).

Scanning electron microscopy (SEM): SEM analyses were made in the "*Servicio Interdepartamental de Investigación*" (SIdI, UAM). It was performed using a Hitachi S-3000N microscope on samples previously metallized with gold in a sputter Quorum, model Q150T-S.

Visualization of DALGEEs was performed with the help of the Non-Destructive Techniques Laboratory of the National Museum of Natural Science of Madrid (CSIC). They were analysed with FEI QUANTA 200, a SEM that operates with three ways of vacuum: high vacuum, low vacuum (0.08 to 1 torr) and environmental way or ESEM (1 to 20 torr), with secondary electrons detector and backscattering detector. DALGEEs were observed at low vacuum.

2.7.2 Micro-scale general procedure for enzyme immobilization

The general procedure is represented in **Fig. 2.2** (Fernandez-Arrojo et al., 2015). A known amount of the carrier was placed inside a micro-centrifuge filter tube and then washed twice with 500 µL of immobilization buffer to equilibrate the support. The washing step included the mixing of the carrier with the buffer by closing the micro-filter tube and inverting 6 times, followed by a centrifuge spin for the separation of the washing buffer from the solid particles, which remained inside the filter holder. After washing, the carrier was mixed (inside the filter holder) with 500 µL of enzyme solution in the immobilization buffer. After a certain time of incubation (30 min for glyoxal carriers, 5 h for VS-activated silica carriers) at room temperature in a roller mixer (J.P. Selecta, Barcelona, Spain), the filter tubes were

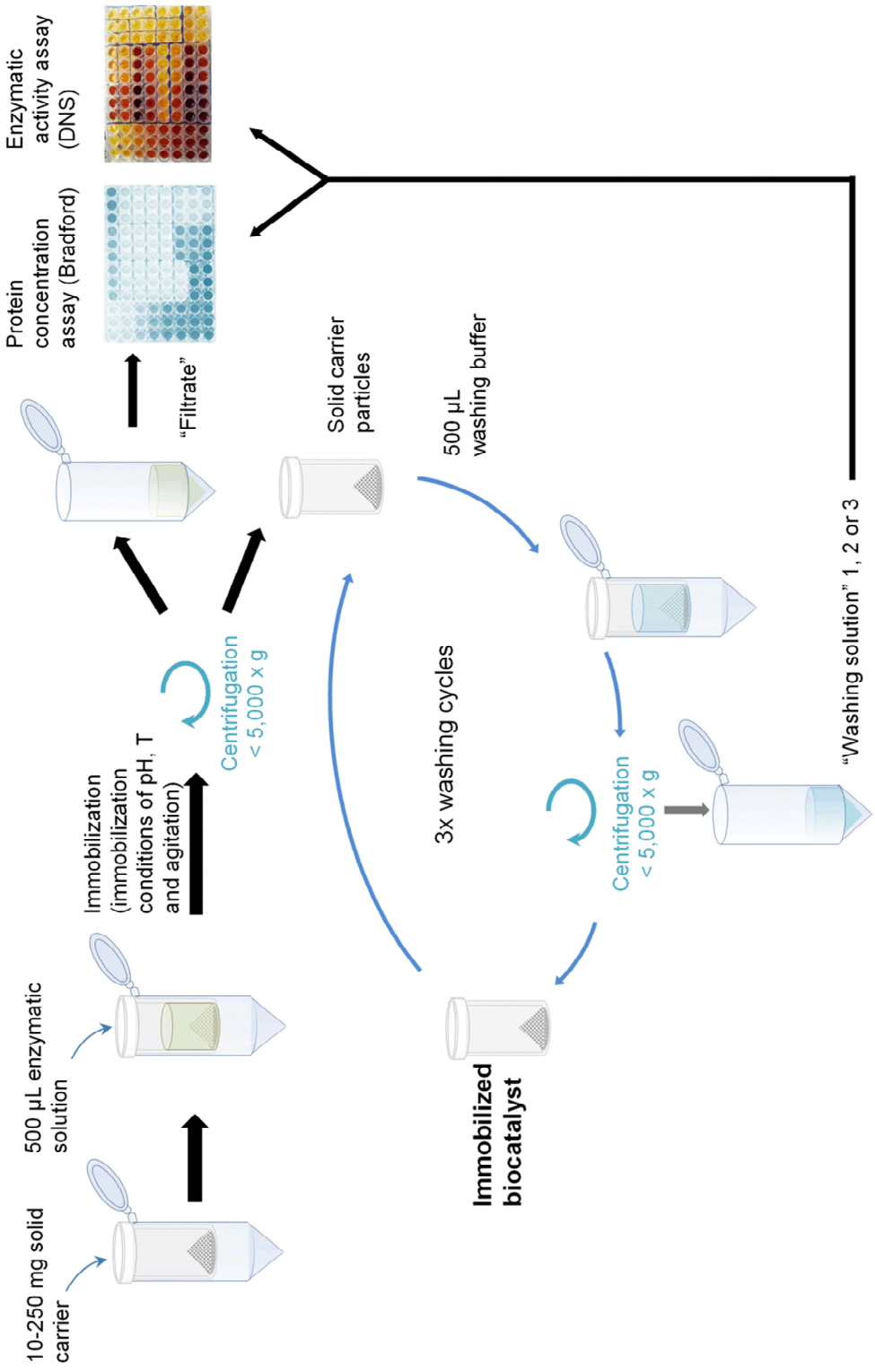


Fig. 2.2. Micro-scale assay to screen the best immobilization conditions.

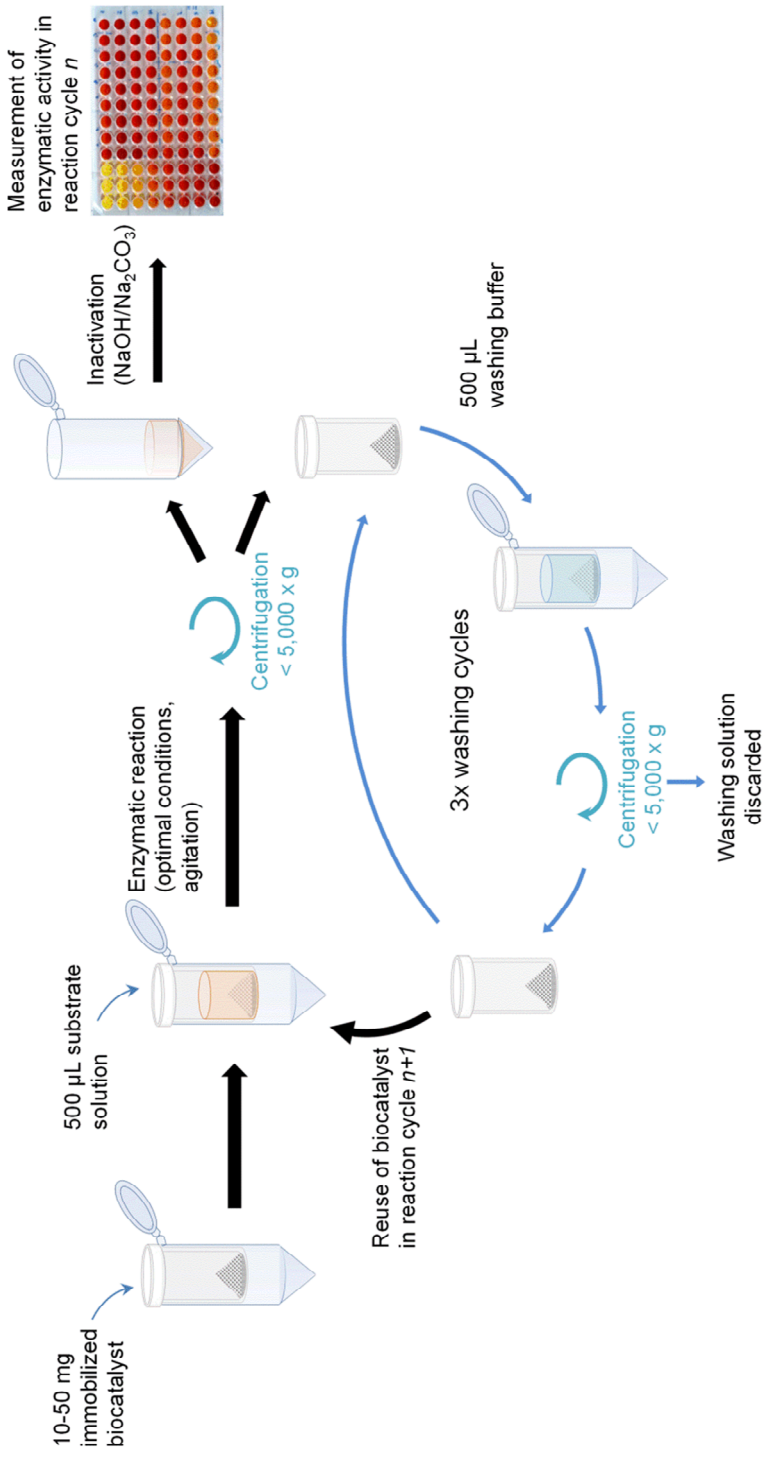


Fig. 2.3. Micro-scale assay for the determination of the apparent activity and operational stability of immobilized biocatalysts.

centrifuged at 5,000 x g and the filtrate was collected and stored. The resulting biocatalyst was washed three times, with 500 µL of reaction buffer and inverting the micro-filter tube 6 times, followed by a filtration step by centrifugation. The washing solutions 1, 2 and 3 were collected and stored at 4°C. The activity and protein concentration of the filtrate and washings was measured as described above. The immobilization yield was determined by subtracting the total protein and enzymatic activity in the filtrate and the washing solutions from the initial values in the enzyme solution.

2.7.3 Entrapment in alginate beads and DALGEE formation

Levansucrase was first crosslinked with transglutaminase (TG). To a 100 mg/mL solution of LEV, 20 U of TG were added and it was incubated for 1 h at 35 ºC with mild agitation. Levansucrase was immobilized by entrapment in 4% (w/v) calcium alginate by dropping with the aid of a peristaltic pump a 1:1 (v/v) solution of enzyme:alginate into a 200 mM $CaCl_2$ solution. The beads were kept with mild agitation for 10 min in this solution and then transferred to a fresh 200 mM $CaCl_2$ solution and kept for another 10 min. Finally, the beads were washed in stability buffer for another 10 min. Immobilization yield was calculated by measuring the activity present in all the the immobilization solutions and comparing it with the initial enzyme solution. The alginate beads were dehydrated with pressurized air to form DALGEEs. This protocol was followed for the immobilization of LEV and LEV after crosslinking with TG.

2.7.4 Micro-scale immobilization of LEV on VS-activated silica

The enzyme was incubated with the support at two different ratios (w/w): 10 and 100 mg of protein per gram of carrier. LEV was previously dissolved in 50 mM potassium phosphate buffer (pH 7.0). The immobilization filter tubes were incubated for 5 h at room temperature with mild agitation. The solid biocatalyst (LEV-VS) was separated of the remaining solution by centrifugation and washed thoroughly with stability buffer. The immobilization yield was determined by subtracting the total protein and enzymatic activity in the filtrate and the washing solutions from the values obtained with the starting enzyme solution.

2.7.5 Apparent activity of the immobilized biocatalysts

The apparent activity is defined as the activity of the biocatalyst measured directly and, unlike the theoretical activity, it takes into account the mass transfer and diffusional restrictions (Worsfold, 1995). The apparent activity of the immobilized biocatalysts was determined by incubating in a micro-centrifuge filter tube a known amount of biocatalyst (20-50 mg) with 500 µL of substrate solution (**Fig. 2.3**). The mixture was incubated at the reaction temperature for 20-30 min in an orbital incubator (Vortemp 1550, LabNet, Edison, NJ, USA) with vigorous (900 rpm) agitation. The reaction mixture was separated from the biocatalyst by centrifugation at 5,000 x g. The supernatant was heated at 96ºC to inactivate the possible lixiviated enzyme. Then, reducing sugars in the supernatant were assayed as described above with the DNS method.

2.7.6 Operational stability micro-scale assay

The operational stability of the biocatalysts was assessed in successive batch reaction cycles (**Fig. 2.3**). Each cycle included the determination, in the micro-centrifuge filter tube, of the apparent activity of the biocatalyst with fresh substrate, followed by three washing steps of the solid with 500 µL of buffer. The relative enzymatic activity of each cycle was referred to the apparent activity of the first cycle. In the case of the DALGEE biocatalysts, the washings between cycles were performed with a 600 g/L sucrose solution to avoid rehydration.

2.7.7 Biochemical characterization of immobilized biocatalysts micro-scale assay

Optimal temperature and pH, enzyme stability and kinetic constants of immobilized biocatalysts were assayed in the same way as for the soluble enzymes but with the aid of filtered centrifuge tubes, weighting a known amount of immobilized biocatalyst.

2.7.8 Large-scale procedure for BAN immobilization

The immobilization of BAN on glyoxal agarose was carried out following the protocol of the supplier with slight modifications. Prior to immobilization, the glyoxal agarose beads were washed with distilled water to remove any preserva-

tives. Simultaneously, contaminants present in the enzymatic preparations were removed with a DP-10 desalting column, and the enzyme was eluted with 100 mM $NaHCO_3$ (pH 10.0), in order to perform immobilization at such pH. The enzyme solution was added to 1 g of glyoxal agarose and incubated for 1 h at room temperature in a roller shaker (JP Selecta S.A., Spain). This immobilization time was optimized taking into account the compromise between the formation of covalent bonds and the stability of the enzyme at pH 10. After this incubation, 10 mg of $NaBH_4$ were added for the reductive amination of the Schiff base bonds and the mixture was incubated at room temperature for 30 min to yield the immobilized biocatalyst BAN-Glx. The immobilization mixture was filtrated with acetate/nitrate cellulose filters (0.45 µm, Merck Millipore, Billerica, MA) to separate the immobilized biocatalyst from the remaining solution. The immobilized biocatalyst was washed thoroughly with 50 mM sodium acetate buffer (pH 5.0) in order to remove any loosely adsorbed enzyme.

2.8 Reactors with immobilized biocatalysts for COS production

2.8.1 Batch reactor

A stirred tank reactor with the immobilized enzyme was set in batch with 1.5 U of immobilized biocatalyst and 100 mL of 1% (w/v) chitosan (CHIT100) in 100 mM acetate buffer (pH 5.0) as substrate. The reactor worked at 50ºC and 400 rpm, with magnetic stirring. Samples were taken at different times and the enzyme was inactivated by heating at 95ºC for 10 min. Viscosity and COS content were analyzed as described above.

2.8.2 Packed-bed reactor

A packed-bed reactor in a glass column (d = 1.5 cm) was set up. Paper filter (cut-off 20-25 μm) was placed at both ends to avoid leakage of the particles. BAN-Glx (1.5 U) was packed by gravity to a length of 1.8 cm and then compressed to 1.5 cm. The column was kept at 50ºC in a water bath. Substrate (partially hydrolyzed chitosan) was pumped using a peristaltic pump at different flow rates. Effect of dilution rate on productivity, conversion yield and continuous production of COS was investigated. Different substrate flow rates of 50% hydrolyzed 1% (w/v) chitosan (previously produced in a batch reactor with BAN-Glx) were pumped through the PBR with a peristaltic pump. When the system was considered to be in steady state, samples were taken and analyzed by HPAEC-PAD.

2.9 Bioactivity assays

2.9.1 Scavenging of ABTS radical

Scavenging of ABTS radical cation was assayed as described by Re and cols. (Re et al., 1999) with slight modifications. The radical was produced as follows: 7 mM ABTS was dissolved in water and mixed with a 2.45 mM (final concentration) potassium persulfate solution. The reaction was kept in the dark at room temperature overnight. The solution was diluted with water to a final absorbance of 0.7 at a wave length of 734 nm and equilibrated at 30ºC. This mixture was stable for at least two days at room temperature.

Different concentrations of COS and FOS (0-30 mg/mL) were tested by adding 20 µL of these solutions to 230 µL of ABTS radical. The absorbance was followed at 734 nm for one hour. Concentration of COS or FOS was represented *vs.* the decrease in absorbance, in percentage. Samples were measured at least 3 times and data was expressed as the media ± standard deviation.

Half maximal scavenging concentration (SC_{50}) was defined as the concentration of a substance, in mg/mL, that is able to produce a 50% decrease in the absorbance of ABTS radical cation in 10 min.

2.9.2 Neuroprotective activity in SH-S5Y5 neuron cultures

These experiments were performed in collaboration with the group of Dr. Juan Carlos Morales in Instituto de Parasitología y Biomedicina "López - Neyra" (IPBLN, CSIC).

Cell cultures: SH-S5Y5 neurons were cultured in collagen-pretreated petri-dishes with DMEM-F12 medium supplemented with Penicillin/Streptomycin and 10% inactivated fetal bovine serum (iFBS).

Cell viability assays: Neuron assays were done in collagen-pretreated 96 well plates by seeding 2 x 10^4 neurons per well in a 100 µL volume and with 24 h of incubation time before compound addition. Tested compounds dissolved in DMSO were then added at different concentrations (2, 0.2 and 0.02 mg/mL) to determine compound toxicity. Final DMSO percentage in each cell was adjusted to 1%

DMSO. Cell viability was evaluated 24 h after compound addition by mitochondrial MTT assay, according to manufacturer.

Neuroprotection assay: Neurons were cultured and plated as described in the cell viability assay. Tested compounds dissolved in DMSO were added at different concentrations (2, 0.2 and 0.02 mg/mL) and after 10 min incubation 100 µM of hydrogen peroxide was added. Final DMSO percentage in each cell was adjusted to 1% DMSO. Cell viability was evaluated 24 hours after compound addition by mitochondrial MTT assay, according to manufacturer.

Statistical analysis: Data are expressed as means ± standard error (SE), with n=8. ANOVA on ranks and post-hoc Dunn's Method were used to find differences between groups. Statistical analysis was performed with SigmaPlot 13.0 and were considered significant when $p < 0.05$.

3 RESULTS & DISCUSSION

3.1 FOS production by levansucrase from *Z. mobilis*

3.1.1 Biochemical characterization of levansucrase

The levansucrase from *Zymomonas mobilis* employed in this work presented a major band of approximately 45 kDa in SDS-PAGE gel (Appendix I, **Fig. A1.1**), in accordance with previous reports (Vigants et al., 2001). A zymogram showed that the enzymatic preparation had three different bands with the ability to produce reducing sugars. An N-terminal analysis demonstrated that the three proteins were isoforms of the levansucrase from *Z. mobilis*. Therefore, the only activity acting on sucrose present in the preparation was levansucrase.

The optimal conditions of temperature and pH were assayed. Levansucrase displayed its maximum activity at 40ºC and pH 5.4 (**Figs. 3.1A** and **3.1B**, respectively), which was in agreement with previous studies with this enzyme (Yanase et al., 1992).

The thermal and pH stability of levansucrase was also studied. After 24 h incubations, the residual activity was determined by the standard activity assay. We observed that the enzyme was stable at temperatures between 4 and 45ºC and pH 3-7 (**Fig. 3.2**). It is worth noting that the enzyme was slightly activated upon incubation at 35°C and also at pH 4.0. In the last case, activation could be related to the formation of ordered microfibril structures at acid pH as described by Goldman et al. (Goldman et al., 2008).

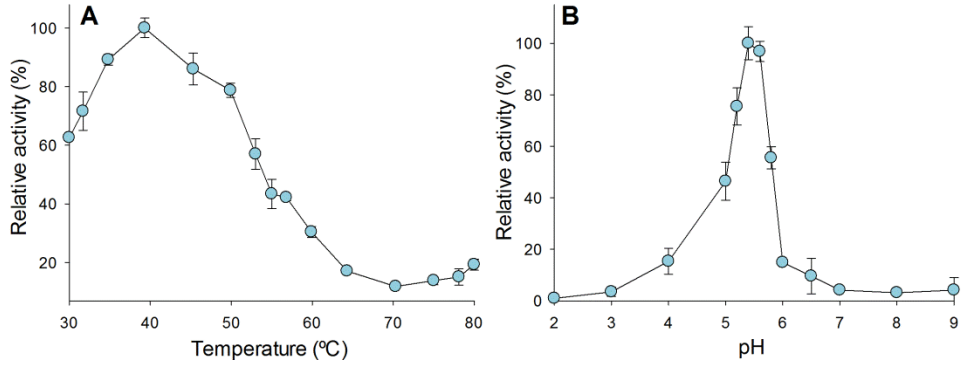


Fig. 3.1. (**A**) Temperature-activity and (**B**) pH-activity profiles of levansucrase from *Z. mobilis*. The relative activity refers to the maximum activity at the optimal conditions of temperature and pH, respectively.

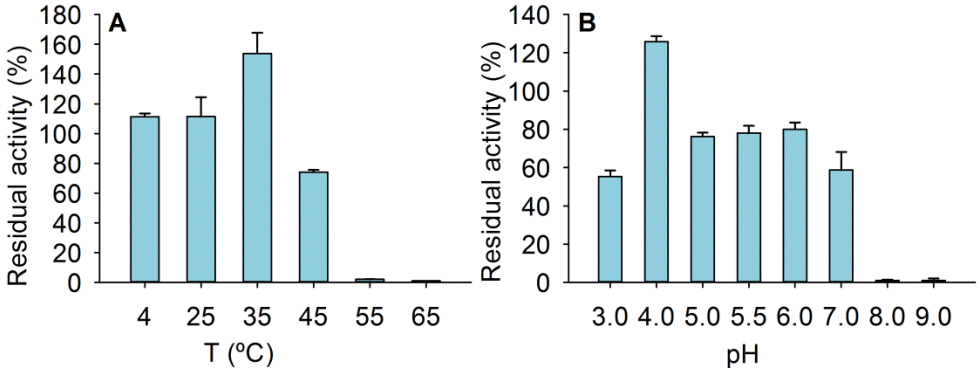


Fig. 3.2. Levansucrase stability towards: (**A**) temperature; (**B**) pH. Levansucrase was incubated under the different conditions of temperature (at pH 5.4) and pH (at 30°C) for 24 h. Residual activity was referred to the initial activity of the enzyme, prior to any incubation, using the standard activity assay with sucrose as substrate.

3.1.2 Formation of levan and FOS by levansucrase

As described in Chapter 1.2.3, levansucrases display three different catalytic activities. First, they form levan by the transfer of the fructosyl moiety of sucrose to a growing polymer chain, releasing glucose as side product (Olvera et al., 2012). Secondly, the fructosyl group can be also transferred to small carbohydrates acting as acceptors (e.g. glucose, sucrose) yielding fructooligosaccharides (FOS) with β(2→6) bonds. Finally, water can also act as nucleophile acceptor of the fructosyl-levansucrase intermediate releasing free fructose (hydrolysis). The balance between the three reactions strongly depends on the nature of the enzyme and on the experimental conditions (Tian and Karboune, 2012).

We analyzed the synthesis of levan by size exclusion chromatography (SEC-HPLC) under different sucrose concentrations (100 g/L and 600 g/L) and temperatures (4 and 40ºC) at pH 5.4 using 5 U/mL of levansucrase (**Fig. 3.3**). SEC analysis showed that the highest yield of levan was obtained at 4ºC and 100 g/L sucrose (**Fig. 3.3A**). The synthesized levan presented the same retention time than the commercial levan from *Z. mobilis* used as standard (data not shown). Based on the chromatographic mobility, the molecular weight (MW) of the formed levan was greater than that of the dextran of higher MW used as standard (144 kDa). In this context, the MW of the levan synthesized by other bacterial levansucrases, e.g. from *Bacillus subtilis*, is at least of 2,000 kDa (reaching values of 100 million Da) with a minimum of 10,000 fructosyl moieties (Tanaka et al., 1980). The levan

obtained at 40ºC showed a slightly higher retention time than its homologous formed at 4ºC, which indicated a lower degree of polymerization at higher temperature. At 4ºC and 600 g/L sucrose, the levan peak displayed also a shoulder that could be related with the presence of a component of lower molecular weight in the biopolymer fraction (**Fig. 3.3C**). This result was in accordance with Vigants and cols. (Vigants et al., 2013), who described that levansucrase is more likely to form longer polysaccharides at lower temperatures.

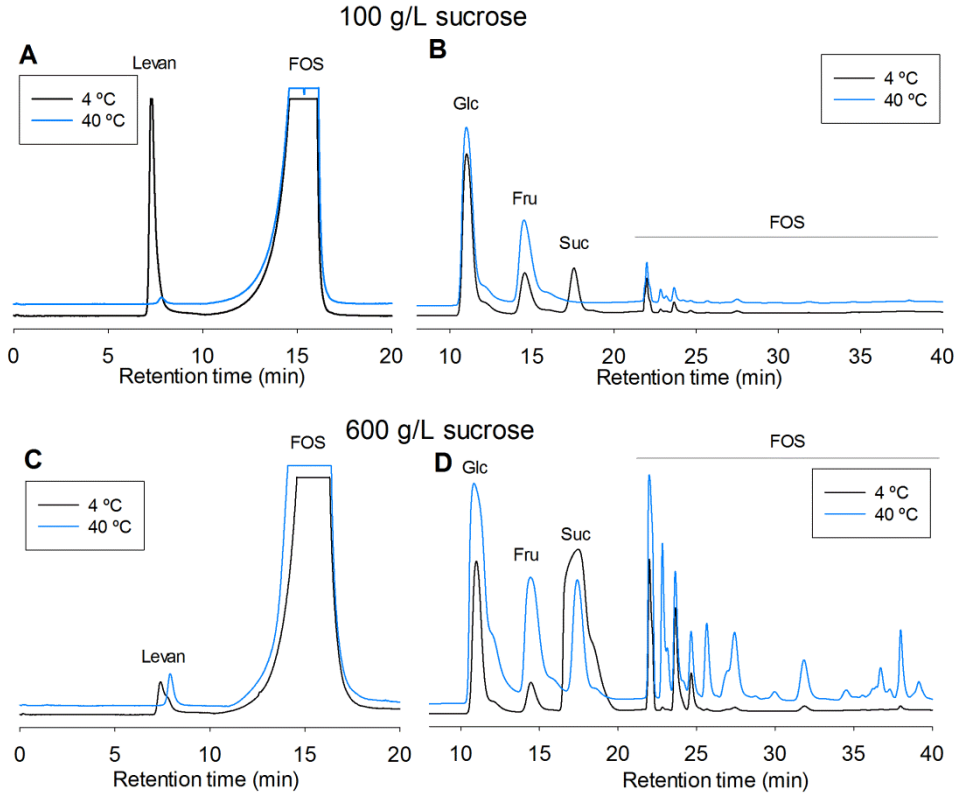


Fig. 3.3. (**A**) SEC chromatogram and (**B**) HPAEC-PAD chromatogram of the reaction mixture after 24 h incubation of levansucrase with 100 g/L sucrose at 4ºC (thick line) and 40ºC (blue line). (**C**) SEC chromatogram and (**D**) HPAEC-PAD chromatogram of the reaction after 24 h incubation of levansucrase with 600 g/L sucrose at 4ºC and 40ºC. Glc: glucose; Fru: fructose; Suc: sucrose; FOS: fructooligosaccharides.

The reaction mixtures were also analyzed by HPAEC-PAD to visualize the formation of FOS and the hydrolysis of sucrose under the same conditions (**Figs. 3.3B** and **3.3D** for 100 g/L and 600 g/L sucrose respectively). At 100 g/L, the low-molecular-weight fraction was formed basically by glucose (which is a by-product of both transfructosylation and hydrolysis reactions), fructose (released only in the

hydrolysis reaction) and the remaining sucrose. Several FOS appeared in the chromatogram, but their concentration was significantly lower than that of the mono- and disaccharides. However, at 600 g/L of sucrose, the FOS concentration substantially increased (**Fig. 3.3D**). A feasible explanation is that a competition is established between the synthesis of short FOS and the formation of high-molecular-weight levan. At high sucrose concentrations, the role of sucrose as acceptor of the fructosyl-enzyme intermediate is favoured and thus short-chain oligosaccharides are synthesized. At lower sucrose concentrations, the growing levan chain has less competence to accept the fructosyl moieties of the fructosyl-enzyme intermediate. Similar results have been obtained with related enzymes such as dextransucrases in the competitive formation of glucooligosaccharides and dextran (Alcalde et al., 1999, Gomez de Segura et al., 2003).

Fig. 3.3D clearly shows that higher temperatures increased the formation of FOS. This result correlates well with the previous observation that lower temperatures yielded levans of higher size. The differences in FOS formation between the two temperatures when 100 g/L sucrose was used as substrate were not so evident compared with the experiments carried out with 600 g/L sucrose.

We measured the concentrations of glucose, fructose and sucrose after 24 h reaction in order to analyze the transglycosylation to hydrolysis ratio under the different experimental conditions. Hydrolytic activity (H) was quantified by the concentration of free fructose. Transfructosylating activity (T) was calculated by the difference between the concentration of free glucose and fructose. The transfructosylation to hydrolysis (T/H) ratio was calculated dividing both activities. **Table 3.1** summarizes the T/H values and the percentage of sucrose consumed after 24 h reaction. Sucrose conversion was notably higher at 40ºC, as expected from the optimum temperature of this enzyme (**Fig. 3.1A**). Interestingly, the T/H ratio was about 4-fold higher at 4ºC compared with 40ºC, which was in accordance with previous reports (Yanase et al., 1992, Jang et al., 2001). Furthermore, the T/H ratio was higher when sucrose concentration increased, because this disaccharide competes with water for the fructosyl-enzyme intermediate.

Table 3.1. Transfrutosylation to hydrolysis ratio (T/H). Comparison of the four different conditions studied for the formation of levan. Data obtained after 24 h reaction with 5 U/mL of levansucrase.

T (ºC)	[Suc] (g/L)	T/H	Sucrose conversion (%)
4	100	3.8	93.4
4	600	4.2	32.8
40	100	0.6	99.9
40	600	1.2	99.5

3.1.3 Analysis of the fructooligosaccharide fraction by HPAEC-PAD

The formation of fructooligosaccharides by levansucrase under optimal conditions (600 g/L sucrose, 40ºC) was studied in detail. The reaction mixtures after 50 h were first analyzed by HPAEC-PAD with a CarboPack PA200 column, which exhibits a good resolution in a broad range of polymerization degree (up to approx. DP 60). Commercial inulin was used as a standard (**Fig. 3.4A**, blue chromatogram), which showed major GFn peaks [*n* fructoses with β(2→1) bonds and a terminal glucose] and small Fn peaks [*n* fructoses with β(2→1) bonds]. **Fig. 3.4A** shows that, in the reaction of levansucrase with sucrose, peaks equivalent in size up to the GF20 of inulin (approx. 3.78 kDa) can be visualized, which illustrates how the levan chain gradually grows. However, the main products obtained under these conditions were small carbohydrates, basically di-, tri- and tetrasaccharides.

In order to analyze the composition of the major FOS in the mixture, we analyzed the same sample with a CarboPack PA1 column (**Fig. 3.4B**) that gives a better resolution in the low MW region. With the use of standards, several FOS were identified: 1-kestose (peak 5), blastose (6), 6-kestose (8), neokestose (10), nystose (12), neonystose (14) and ^{1}F-fructosylnystose (18). Other products not coeluting with any of the available standards could not be identified.

Fig. 3.5 illustrates the time course of the formation of the main acceptor products (the three kestoses and the tetrasaccharide nystose). At the beginning of the reaction the most abundant oligosaccharide was 6-kestose, reaching a

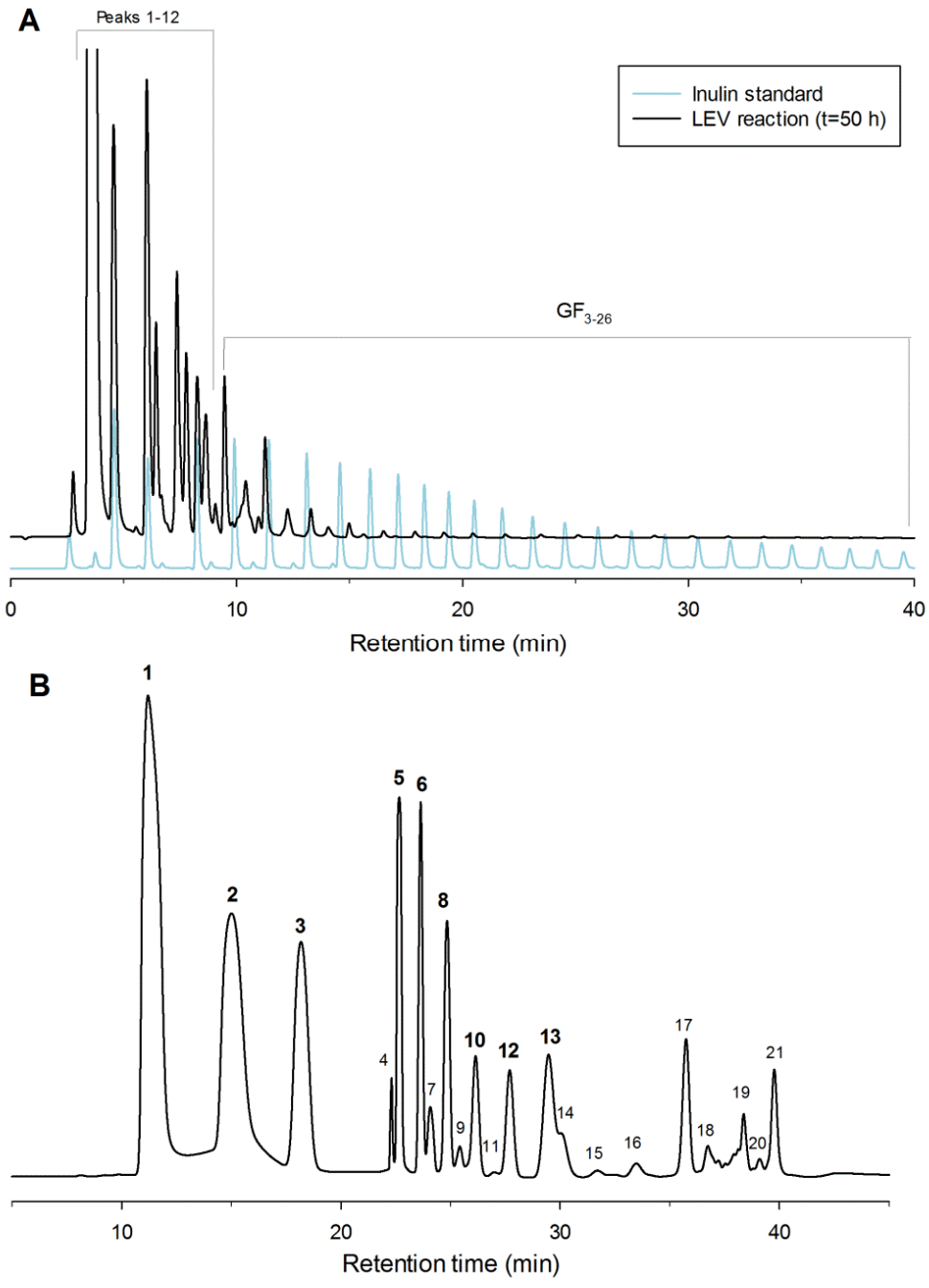


Fig. 3.4. (**A**) HPAEC-PAD chromatogram with CarboPack PA200 column (black chromatogram) of the fructooligosaccharides profile after 50 h reaction of LEV with 600 g/L sucrose at 40ºC. (Blue) Inulin standard. GFn: n fructoses with β(2→1) linkages and a terminal glucose. (**B**) HPAEC-PAD chromatogram with CarboPack PA1 column of the fructooligosaccharides synthesized by LEV with 600 g/L sucrose after 18 h reaction at 40ºC. Peak identification: (**1**) glucose; (**2**) fructose; (**3**) sucrose; (**5**) 1-kestose; (**6**) blastose; (**8**) 6-kestose; (**10**) neokestose; (**12**) nystose; (**13**) isolated tetrasaccharide. Unknown peaks: 4,7,9,11,14-21.

maximum at 6 h. Afterwards the concentration of 6-kestose diminished as it was used as scaffold to elongate the levan chain. In contrast, 1-kestose accumulated progressively. It is also noteworthy the presence of blastose [Fru-β(2→6)-Glc] in the reaction mixtures. This sucrose isomer was previously reported in the reaction of sucrose with the levansucrase from *B. megaterium*; the authors demonstrated that it was formed by the hydrolysis of the synthesized neokestose (Homann et al., 2007). In our work, blastose accumulated whereas neokestose concentration decreased (**Fig. 3.5,** yellow diamonds) confirming such hypothesis, although it was not possible to rule out that blastose could be synthesized by the acceptor reaction with glucose.

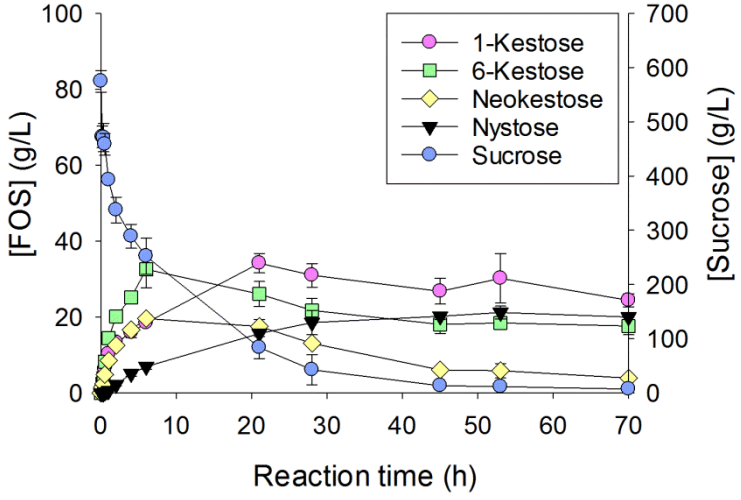


Fig. 3.5. Reaction of LEV with 600 g/L sucrose at pH 5.4 and 40ºC. (**A**) Time progress of known FOS concentration (left axis) and sucrose concentration (right axis). (**B**) Composition of the reaction mixture (in weight percentage) at the point of maximum FOS concentration (21 h).

Production of known FOS by levansucrase reached its maximum when 85% of sucrose was consumed (21 h of reaction). At this point of the reaction the mixture was made up of 31% glucose, 14% fructose, 15% sucrose, 15% of quantified FOS (for which standards were available) and 25% of unknown products (including the small amount of levan, **Fig. 3.3C**). The concentration of unknown products was estimated as the difference between initial sucrose concentration and the sum of known products.

3.1.4 Identification of a tetrasaccharide synthesized by levansucrase

We isolated by semipreparative HPLC peak 13 in **Fig. 3.4B**, which was one of the unknown FOS obtained at higher concentration with levansucrase. The compound was purified and characterized by mass spectrometry and NMR. Mass spectrometry showed that it was a tetrasaccharide (MW = 666 g/mol, see Appendix I, **Fig. A2.1**). The 1D and 2D NMR spectra displayed one anomeric signal, arising from the glucose residue and several signal group patterns, recognizable as fructose moieties. From the analysis of the signals present in the COSY, NOESY, HSQC, HSQC-TOCSY and HMBC spectra, the full assignment of the 1H and 13C resonance signals belonging to the different residues was achieved (Appendix I, **Fig. A2.2**). Thus, the NMR data permitted to unambiguously identify peak 13 as 6,6-nystose [Fru-β(2→6)-Fru-β(2→6)-Fru-β(2→1)-αGlc], a member of the levan-type FOS.

3.2. Immobilization of levansucrase

There are many fructosyltransferases and β-fructofuranosidases that have been immobilized for the production of inulin-type FOS from sucrose (Plou et al., 2014, Singh et al., 2016). However, production of levan and levan-type FOS has been less studied (Appendix I, **Table A4.1**). In this Thesis, two immobilization methods were assessed: immobilization by entrapment in calcium alginate beads (Santos-Moriano et al., 2015b) and covalent immobilization on vinyl sulfone-activated silica carriers (Santos-Moriano et al., 2016a).

3.2.1 Entrapment of levansucrase in calcium alginate

Levansucrase was first immobilized by entrapment in alginate beads followed by further dehydration for the formation of DALGEEs (Dry ALGinate Entrapped Enzymes) (Fernandez-Arrojo et al., 2013). The enzyme was both immobilized on its own and after crosslinking with transglutaminase (TG) to minimize the leakage of the enzyme from the polymeric matrix. Both strategies showed more than 90% theoretical immobilization yield (**Table 3.2**). However, the apparent (experimental) activity of the biocatalysts (42 U/g in the case of LEV and 38 U/g in the case of LEV-TG) was much lower than the theoretical values, probably due to diffusional limitations caused by the dehydration process. Moreover, the enzyme seemed to lose activity when it was crosslinked with transglutaminase, maybe due to rigidification caused by the formation of covalent bonds.

Table 3.2. Immobilization of levansucrase from *Z. mobilis* by entrapment in DALGEEs

		Theoretical[a]		Apparent[b]	
Biocatalyst	Initial (U)	Activity (U/g)	Yield (%)	Activity (U/g)	Yield (%)
LEV	1900 ± 140	3500 ± 150	91 ± 10	42 ± 0.1	1.10 ± 0.07
LEV + TG	1800 ± 220	2800 ± 230	96 ± 18	38 ± 0.7	1.0 ± 0.13

[a] Theoretical values determined by subtracting the total initial activity prior to immobilization and the remaining activity in the filtrate and washing solutions after immobilization.
[b] Experimental activity (standard assay).

Comparing the operational stability of both biocatalysts (with and without TG crosslinking prior to immobilization) the crosslinked DALGEEs worked significantly

better in a sequence of reactions in batch (**Fig. 3.6**), maintaining about 80% of the initial activity after 14 reaction cycles. Despite the significant operational stability, other methods were explored for the immobilization of LEV trying to achieve a higher recovery and apparent activity.

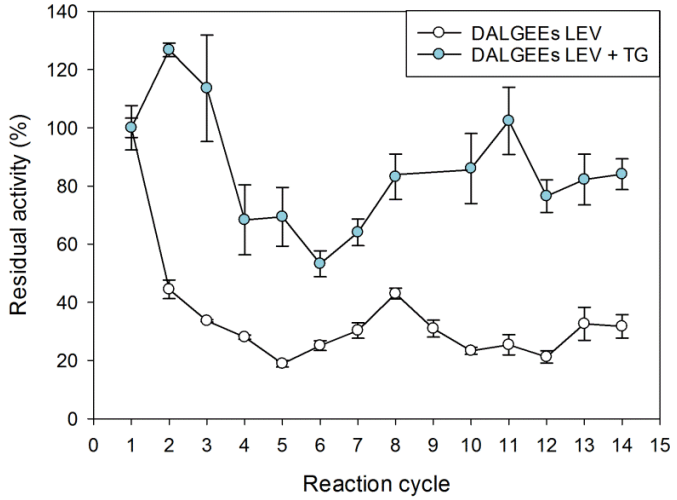


Fig. 3.6. Operational stability of levansucrase entrapped in DALGEEs with (blue circles) and without (white circles) crosslinking with transglutaminase. Values are referred to the activity of each biocatalyst in the first cycle.

3.2.2 Covalent immobilization of levansucrase in vinyl sulfone-activated silica

Unfortunately, conventional methods for covalent immobilization involve incubations in alkaline conditions to ensure the formation of covalent bonds, and this enzyme is not stable at pH above 7 (**Fig. 3.2B**). The activation of carriers with divinyl sulfone (DVS) for enzyme immobilization was first described by Bryjak et al. (Bryjak et al., 2008) for an organic support (cellulose) and by Ortega-Muñoz et al., (Ortega-Muñoz et al., 2010, Morales-Sanfrutos et al., 2010) for an inorganic material (silica). This approach has been applied to other supports like agarose (dos Santos et al., 2015a, dos Santos et al., 2015b). VS-activated carriers enable covalent coupling at neutral pH, and have been reported to bind several enzymes and proteins even under moderate acidic conditions (dos Santos et al., 2015a, dos Santos et al., 2015b, Lopez-Jaramillo et al., 2012, Ortega-Muñoz et al., 2010). The covalent bonds between the enzyme and the support are formed by means of a Michael-type addition that involves the amino groups of lysines, the imidazole moieties of histidines and/or the sulfhydryl group of cysteines (**Fig. 3.7**).

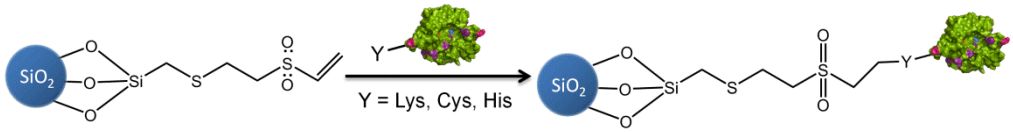


Fig. 3.7. Enzyme immobilization mechanism on VS-activated silica.

3.2.2.1 Characterization of vinyl sulfone-activated silica

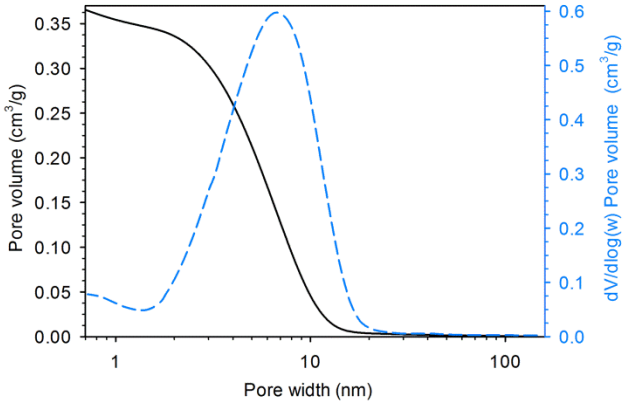
Silica was selected as carrier due to its extraordinary chemical, mechanical and biological stability, as well as its resistance to large changes in pressure and flow-rates in different reactors (Ortega-Muñoz et al., 2010). Activation of silica with DVS was carried out as described elsewhere (Morales-Sanfrutos et al., 2010). The morphology and porous structure of VS-activated silica was visualized by SEM, showing that the particles have not visible porous at 1,000x (Appendix I, **Fig. A3.1**).

Fig. 3.8 represents the pore size distribution of this support calculated using the BJH method (Barrett et al., 1951) and summarizes the main textural properties of VS-activated silica. It is basically a mesoporous material, with an average pore size close to 7 nm. The contribution of the micropore region was negligible. Considering that this enzyme is about 5 x 6 x 7 nm in diameter (see 3D model in **Fig. 3.9**), the above data indicates that levansucrase can hardly diffuse into the pores, and the enzyme must be basically immobilized on the external surface of the silica particles.

3.2.2.2 Micro-scale immobilization of levansucrase on VS-activated silica

Taking into account the thermal and pH stability of the enzyme (**Fig. 3.2**), neutral pH and room temperature were selected as the immobilization conditions to bind levansucrase to VS-activated silica. LEV was immobilized on VS-activated silica carriers at pH 7.0 using two different protein loadings (10 mg/g and 100 mg/g support) following the micro-scale protocol described in Chapter 2.7.2. Using a loading of 10 mg of protein per gram of support the theoretical yield, calculated by measuring the remaining protein and activity in the filtrate and washing solutions, was 89% for protein and 96% for activity (**Table 3.3**). These results indicated that it was possible to successfully attach, at neutral pH, an enzyme that is intrinsically unstable at alkaline pH. It is worth noting that covalent immobilization on VS-activated silica was completed in only 5 h; it is a significantly shorter time than the

required for other materials such as epoxy-activated supports (48-72 h) (Berrio et al., 2007, Hill et al., 2016).



BET surface area (m^2/g)	Pore volume (cm^3/g)	Average pore size (nm)
242	0.36	6.8

Fig. 3.8. BJH adsorption cumulative pore volume (solid black) and pore distribution (dash blue) of vinyl sulfone-activated silica. Table: textural data of this support obtained from nitrogen adsorption isotherms.

Table 3.3. Immobilization of levansucrase from *Z. mobilis* on vinyl-sulfone silica.

Protein		Activity				
Initial loading (mg/g)	Yield[a] (%)	Initial[b] (U)	Theoretical[a]		Apparent[c]	
			Activity (U/g)	Yield (%)	Activity (U/g)	Yield (%)
10	89 ± 4	8.0 ± 0.5	152 ± 11	96 ± 13	40 ± 15	25 ± 9
100	83 ± 4	133 ± 0.4	2500 ± 100	93 ± 7	185 ± 5	7 ± 0.2

[a] Theoretical values determined by subtracting the total initial protein or activity prior to immobilization and the remaining protein or activity in the filtrate and washing solutions after immobilization.
[b] Measured in a control of soluble levansucrase after incubation (5 h) at the immobilization conditions.
[c] Experimental activity (standard assay).

Other covalent immobilization methods that can be carried out at neutral pH are characterized by a lower efficiency than this one involving vinyl sulfone activation. Glutaraldehyde-assisted strategies are also very fast but the high reactivity of this molecule usually causes a notable loss of activity. Methods that involve carboxylic acids of the protein typically employ the carbodiimide chemistry,

followed by reaction with nucleophiles on the surface of the carrier (Gao and Kyratzis, 2008). Such protocols often lead to a low recovery of activity, partly because carboxyl groups are essential for the catalytic machinery of numerous enzymes, and in particular of glycosidases. In addition, the low stability of O-acylisourea and N-hydroxysuccinimide ester intermediates in aqueous solution contribute to the modest coupling efficiency (Homaei et al., 2013).

It is well known that enzyme reactivity depends on the number and accessibility of reactive groups. The levansucrase from *Z. mobilis* contains 14 lysines, 9 histidines and 3 free cysteines in its amino acid sequence (GenBank: AAA27702.1). With this sequence, modelling of the 3D structure of levansucrase from *Z. mobilis* was performed with Swissmodel, using the 3D structure of levansucrase from *B. subtilis* (PDB: 1OYG, 24% identity) as a template. The model shows that most of the Lys (7 residues) and several histidines are located on the enzyme surface ready to react with the vinyl sulfone groups of the carrier (**Fig. 3.9**), which could explain the efficient coupling.

Although the theoretical immobilization yields were quite high, the apparent activities (experimental) of the immobilized biocatalysts LEV-VS, measured by the DNS method, were 185 U/g (100 mg/g) and 40 U/g (10 mg/g), with apparent yields of 7% and 25%, respectively (**Table 3.3**). The apparent activity is affected by mass transfer and diffusional limitations (Worsfold, 1995), typically causing a decrease of the activity displayed by the enzyme. However, the small pore size of VS-activated silica seems to exclude mass diffusion constraints in this case. On the other hand, the presence of Lys or His residues next to the catalytic center could lead to the binding certain enzyme molecules in an unproductive way. In addition, the rigidification of the enzyme caused by the formation of covalent bonds between the enzyme and the support could also contribute to a decrease in activity. Nonetheless, the activity displayed by LEV-VS biocatalyst and the experimental recovery of activity were significantly higher than the obtained with by entrapment in DALGEEs (**Table 3.3**). This could be related, as stated before, with diffusional restrictions through the polymeric matrix of the DALGEEs. Regarding LEV-VS,

these diffusional limitations must be minimized because the enzyme is mostly bound on the surface of silica.

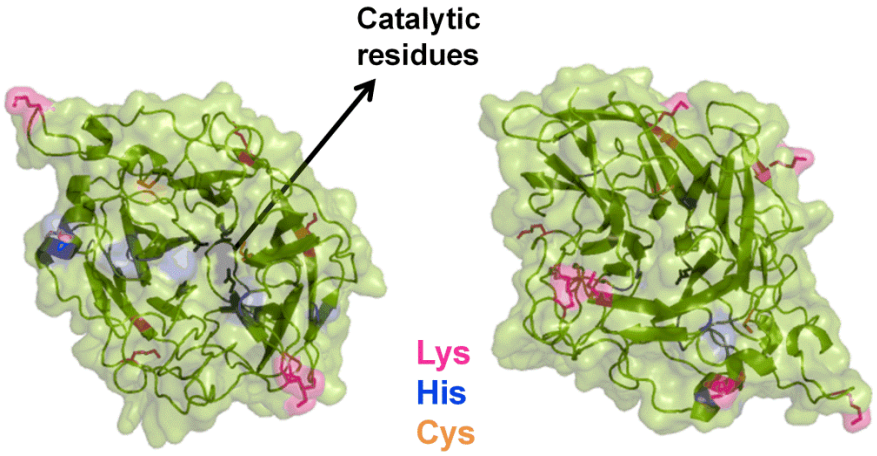


Fig. 3.9. 3D-structure model of levansucrase from *Z. mobilis* obtained with Swissmodel. **Left**: front view of the protein showing the active site (black). **Right**: back view showing some Lys and His residues on the surface. Residues more likely to react with the sulfone groups of the carrier are in pink (Lys), blue (His) and orange (Cys).

This loss of activity upon covalent immobilization was in accordance with the kinetic parameters of the soluble and immobilized enzymes measured under the standard conditions with sucrose (Appendix I, **Fig. A5.1**). The K_M values for free and covalently-attached enzymes were 100 ± 11 and 87 ± 12 mM, respectively, indicating that the affinity of immobilized enzyme for sucrose is hardly affected. In contrast, V_{max} decreased from 1265 ± 45 to 117 ± 4 μmol mg^{-1} min^{-1} as a consequence of immobilization. These values indicate that the insolubilization of the enzyme mainly affects to the rate of transformation of substrate into products.

3.2.2.3 Operational stability of LEV-VS in batch

In order to assess the operational stability of the immobilized biocatalyst, LEV-VS was used in a series of batch reactions with 100 g/L sucrose as substrate, following the micro-scale protocol. After each reaction cycle (20 min), the biocatalyst was thoroughly washed with buffer to remove residual substrate and products, as well as any desorbed enzyme. After 17 cycles, LEV-SV conserved around 50% of its initial activity (**Fig. 3.10**). After an approximate 40% loss of activity in the first 4 cycles, the activity of LEV-VS remained quite stable indicating

that the biocatalyst can be suitable for its repeated use in batch reactors. This loss of activity in the first cycles is typical of covalent immobilizations studies (Martin et al., 2003, Azevedo et al., 2015, Binay et al., 2016), and can be a consequence of the desorption of enzyme molecules that are non-covalently attached to the carrier –which is favoured by the polarity of sugar solutions– or the existence of different populations of enzyme molecules differing in the number of covalent bonds with the support matrix and thus in their intrinsic stability.

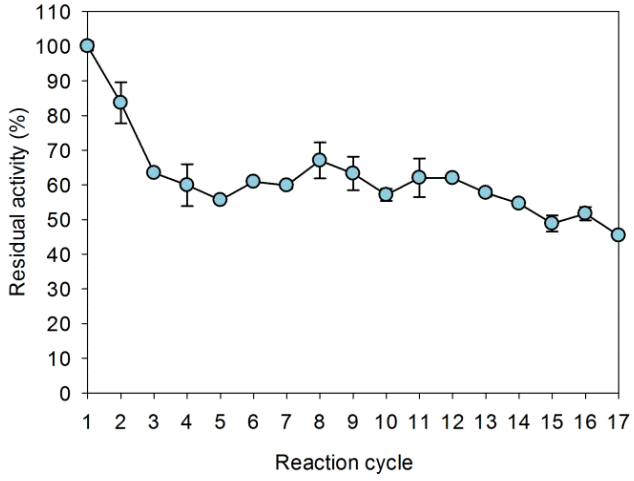


Fig. 3.10. Operational stability of LEV-VS after a series of reactions in batch. Values are referred to the activity of LEV-VS in the first cycle.

3.2.2.4 Production of fructooligosaccharides with LEV-VS

The production of fructooligosaccharides and levan by LEV-VS was compared with that of the free enzyme. In the case of soluble levansucrase, we observed in Chapter 3.1 that depending on the reaction conditions –mainly temperature and substrate concentration–, the enzyme produced preferably levan or FOS (Santos-Moriano et al., 2015a). At low temperature and low concentration of sucrose, the enzyme synthesized levan in detriment of FOS. On the other hand, using 600 g/L sucrose at 40ºC, the enzyme produced FOS with different linkages (**Fig. 3.3D**, blue chromatogram).

Even under the optimal conditions for levan formation, the immobilized levansucrase on vinyl sulfone did not synthesize levan (data not shown). On the

contrary, the enzyme was able to produce FOS. The formation of FOS by LEV-VS was followed by HPAEC-PAD under the optimal conditions for oligosaccharide synthesis (600 g/L sucrose and 40ºC), and compared with the soluble enzyme (**Fig. 3.11**). Comparing both chromatograms, it can be stated that the reaction with the immobilized levansucrase was more selective, yielding fewer products than with the soluble enzyme. The soluble levansucrase was able to form FOS of the ^{1}F- (inulin-like), ^{6}F- (6-kestose family) and ^{6}G- (neoFOS) series, as well as several unidentified peaks (**Fig. 3.11**, asterisks). On the other hand, LEV-VS was more selective towards the formation of FOS of the β(2→1) type, i.e. 1-kestose and nystose (**Fig. 3.11**, black chromatogram, peaks 4 and 8). This fact could help to explain the negligible levan formation, which would require the production of an homologous series of FOS of the β(2→6) family. The diffusional restrictions within the immobilized biocatalyst could also favor the formation of small oligomers in comparison with long polysaccharides. It cannot be excluded that the local microenvironment on the surface of the activated silica may exert an effect on the selectivity of the process, as reported with other immobilized enzymes (Rodrigues et al., 2013). In fact, a change in the product profile of levansucrase upon immobilization was also observed in the immobilization experiments using the DALGEEs entrapment approach (data not shown) (Santos-Moriano et al., 2015b).

The main products synthesized by LEV-VS were quantified and compared with the soluble enzyme (**Fig. 3.12**). Starting with 600 g/L sucrose, the immobilized enzyme rendered a maximum yield of approximately 95 g/L of identified FOS after 28 h (**Fig. 3.12A**), a value very similar to that obtained with the soluble enzyme, under the same conditions. However, the distribution of the main products varies between both preparations (**Fig. 3.12B**), as the syrup obtained with the immobilized enzyme was enriched in 1-kestose and nystose, whereas the soluble enzyme gave rise to a more heterogeneous mixture. Considering the mass balance, the rest of products must correspond to unidentified FOS and, in the case of the soluble enzyme, to a small amount of levan, as detailed above in Chapter 3.1.

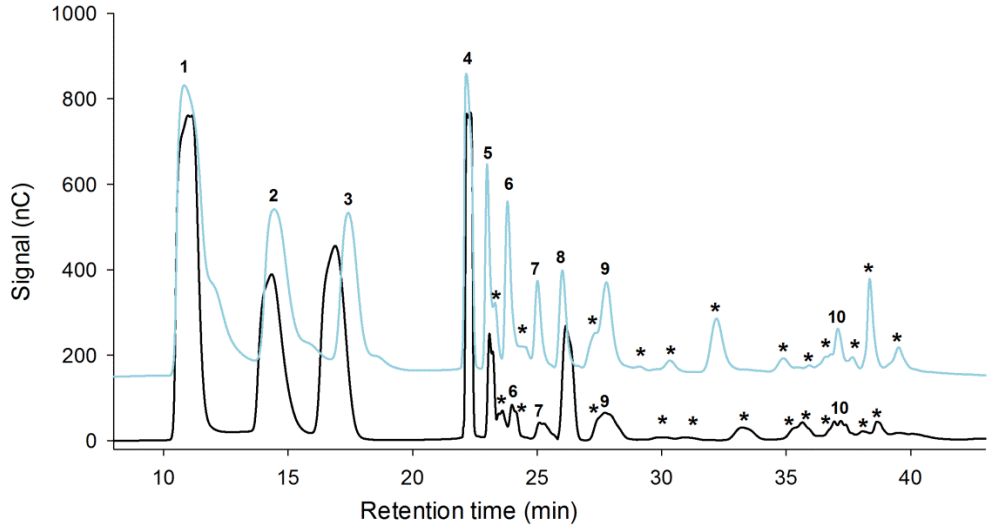


Fig. 3.11. HPAEC-PAD chromatograms of the reaction of sucrose with soluble levansucrase (blue) and immobilized LEV-VS (black). Reaction conditions: 600 g/L sucrose, 50 mM acetate buffer pH 5.4, 5 U/mL enzyme, 40ºC, 28 h. Identified peaks: (**1**) glucose, (**2**) fructose, (**3**) sucrose, (**4**) 1-kestose, (**5**) blastose, (**6**) 6-kestose, (**7**) neokestose, (**8**) nystose, (**9**) 6,6-nystose, (**10**) ^{1}F-fructosylnystose.

Sucrose, fructose and glucose concentrations were measured after 28 h in order to obtain the transfructosylation to hydrolysis ratio for both soluble and immobilized enzymes. The T/H ratio slightly increased upon immobilization (1.7 *vs.* 1.2, see **Table 3.1**), probably due to a change in the microenvironment around the enzyme molecules. However, it still did not reach the levels of the soluble enzyme when incubated at low temperatures.

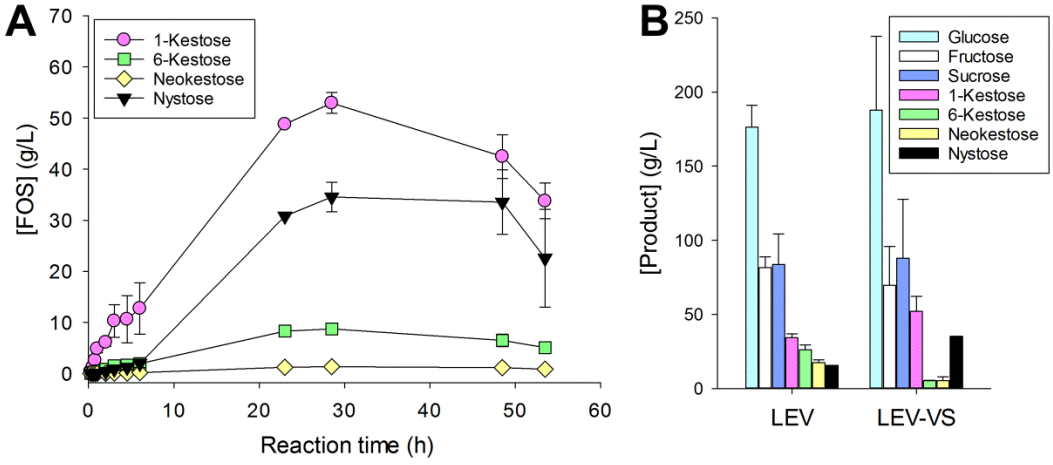


Fig. 3.12. (**A**) Production of identified FOS by immobilized LEV-VS. Reaction conditions: 600 g/L sucrose, 50 mM sodium acetate buffer pH 5.4, 5 U/mL LEV-VS, 40ºC. (**B**) FOS quantification of soluble LEV and LEV-VS reactions at 28 h.

3.3 Production of chitooligosaccharides by commercial enzymes

3.3.1 Chitosanolytic activity screening

Based on previous works that reported the presence of chitosanolytic activity on commercial enzyme preparations (Pantaleone et al., 1992, Montilla et al., 2013, Cabrera and Van Cutsem, 2005), we screened the hydrolytic activity towards chitosan CHIT100 (100-300 kDa, DD ≥ 90%) in several commercial enzymes whose declared activity was pectinase, cellulase (endo-1,4-β-D-glucanase), xylanase or protease (**Table 3.4**). Using the DNS[1] assay for detection of reducing sugars release, the three more active preparations were Rapidase TF (a pectinase from *Aspergillus niger*), Neutrase 0.8L (a protease from *Bacillus amyloliquefaciens*), and BAN 480L (an α-amylase from *B. amyloliquefaciens*), which showed a moderate activity towards chitosan (0.47 ± 0.03, 0.54 ± 0.03, and 3.39 ± 0.13 Units per mL, respectively). Two other pectinolytic preparations (Pectinex Ultra SP-L and Klerzyme 150) and a xylanase (Shearzyme 2X) displayed minor chitosanolytic activity. Therefore, we focused on Rapidase TF, Neutrase 0.8L, and BAN 480L for further experiments (Santos-Moriano et al., 2016b, Santos-Moriano et al., 2017).

Other commercial pectinases from *Aspergillus niger* had been previously reported to display chitosanolytic activity (Cabrera and Van Cutsem, 2005, Abd-Elmohdy et al., 2010); in one case, the pectinase isozyme responsible for the hydrolytic activity towards chitosan was purified (Kittur et al., 2003a). Considering certain degree of similarity between chitosan and pectin (Introduction, **Fig. 1.5** and **Fig. 1.6**) it was not so unexpected for these enzymes to show some activity on chitosan.

Several commercial proteases were also reported to exhibit chitosanolytic activity (Li et al., 2005, Pan et al., 2016, Vishu Kumar and Tharanathan, 2004); however, the presence of such activity in the neutral protease from *B. amylolique-*

[1] DNS assay is based in a redox reaction between the reducing end of sugars and the DNS acid that becomes an aldehyde with red color. Although the method was used successfully to find and measure chitosanolytic activity, we found out later that there was some interference, due to antioxidant activity of COS described later in this Thesis. We are, therefore, underestimating the total activity in the commercial preparations.

faciens (Neutrase 0.8L) had not been described before. In this case it was more risky to state that the chitosanolytic activity came from the protease, although some of the authors of the above publications adventured to do so.

Table 3.4. Screening of chitosanolytic activity in commercial enzyme preparations.

Enzyme	Supplier	Source	Declared activity	Chitosanolytic activity (U/mL)[a]
Alcalase	Novozymes	*Bacillus licheniformis*	Alkaline protease	–
BAN 480L	Novozymes	*Bacillus amyloliquefaciens*	α-amylase	3.39 ± 0.13
E-CELBA	Megazyme	*Bacillus amyloliquefaciens*	β-Glucanase	–
Flavourzyme	Novozymes	*Aspergillus oryzae*	Protease	–
Klerzyme 150	DSM	*Aspergillus niger*	Pectinase	0.016 ± 0.005
Neutrase 0.8 L	Novozymes	*Bacillus amyloliquefaciens*	Neutral protease	0.54 ± 0.03
NovoShape	Novozymes	*Aspergillus oryzae*	Pectin methyl esterase	–
Papain	Sigma-Aldrich	*Carica papaya latex*	Acid protease	–
Pectinex Ultra SP-L	Novozymes	*Aspergillus aculeatus*	Pectinase	0.010 ± 0.004
Pentopan Mono Conc. BG	Novozymes	*Aspergillus oryzae*	Xylanase	–
Rapidase TF	DSM	*Aspergillus niger*	Pectinase/ hemicellulase	0.98 ± 0.07
Shearzyme 2X	Novozymes	*Aspergillus oryzae*	Xylanase	0.007 ± 0.005
Ultraflo L	Novozymes	*Humicola insolens*	β-Glucanase	–

[a] Reaction conditions: 1% (w/v) chitosan CHIT100, 20% (v/v) enzyme, 50°C, 1,400 rpm, pH 5.0, 1 h.

Chitosanolysis with the aid of α-amylase had been reported elsewhere (Zhang et al., 1999, Wu, 2012). Although the authors of these articles claimed that the

enzyme responsible of the hydrolysis of the β(1→4) bonds was the α-amylase itself, they did not present evidence enough to rule out the possibility of contamination with another enzyme in the commercial preparation. In fact, considering that Neutrase and BAN preparations are produced by the same microorganism (*B. amyloliquefaciens*), the fact that the protein responsible for the chitosanolytic activity was the same could not be excluded.

In order to have a deeper insight on the nature of the chitosanolytic activity in the three samples, SDS-PAGE and zymograms were made. Zymograms were made with gels that included glycol-chitosan in their preparation. The high specificity of this kind of enzymes for their substrate can result in bands with some retention with respect to the denaturing gels. That is most likely the case of Rapidase (**Fig. 3.13**, lanes 1 and 2), for which the chitosanolytic band in the zymogram is higher than any band on the SDS-PAGE gel. According to the supplier, this commercial preparation is a mixture of pectinase, hemicellulase and cellulase enzymes, therefore containing many proteins that could harbor the chitosanolytic activity by substrate similarity. Considering what was stated earlier about these enzymes being able to hydrolyse chitosan, and the evidence found in literature about other pectinases from the same microorganism (Kittur et al., 2003b), it seems possible that the pectinase from *A. niger* present in the Rapidase TF preparation catalyzed the hydrolysis of chitosan.

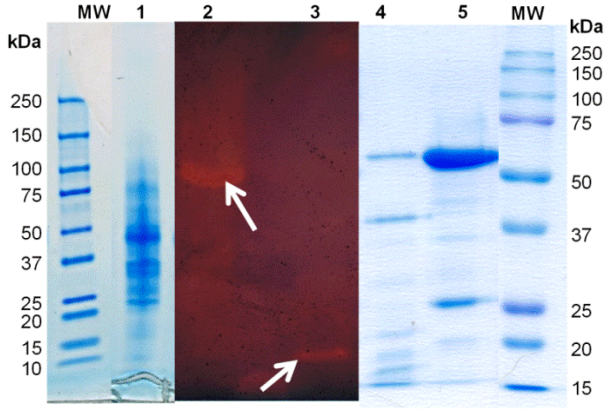


Fig. 3.13. Protein electrophoresis. Lane 1: SDS-PAGE of Rapidase TF; Lane 2: zymogram of Rapidase TF; lane 3: zymogram of Neutrase 0.8L; lane 4: SDS-PAGE of Neutrase 0.8L; lane 5: SDS-PAGE of BAN 480L. MW: molecular weight markers.

On the other hand, in the case of both Neutrase and BAN, we can only conclude that the chitosanolytic activity does not correspond to their major bands in the SDS-PAGE gels. Zymogram could not be done for the BAN preparation, but if we can assume that the enzyme responsible for the chitosanolytic activity in both preparations is the same, then we could compare with the Neutrase zymogram (**Fig. 3.13**, lane 3). The lytic band appeared towards the end of the gel, indicating a very small protein (< 20 kDa). Neutrase protease has a reported MW of 56.7 kDa (**Fig. 3.13**, lane 4), and the α-amylase from BAN has a reported MW of 58.4 kDa (**Fig. 3.13**, lane 5). Although, as explained earlier, SDS-PAGE and zymograms cannot be directly compared, these experiments discarded the presence of chitosanolytic activity in the protease and α-amylase themselves.

3.3.2 Monitoring of chitosan hydrolysis by SEC-HPLC

The hydrolysis of chitosan catalyzed by the three enzymes was followed by SEC-ELSD. **Fig. 3.14** shows the progress of the reaction of chitosan CHIT600 (600-800 kDa, DD ≥ 90%) with Neutrase 0.8L. The high-molecular-weight (HMW) chitosan (peak 1) disappeared after 24 h, which indicated efficient hydrolysis of the substrate. A new peak appeared (peak 2) with the same retention time as a chitosan oligosaccharide standard (MW ≤ 2000). The monomers (GlcN and GlcNAc) and the buffer salts coeluted in peak 3. However, the specific composition of the COS fraction cannot be inferred by this methodology. The reactions using chitosans of different DP and DD gave rise to similar SEC-HPLC profiles with all the enzymes (data not shown). In conclusion, SEC-ELSD analyses confirmed the complete disappearance of the HMW chitosan after 24 h under the assayed conditions.

3.3.3 Production of COS by BAN, Neutrase and Rapidase

The pattern of COS formed by the three selected enzymes was further analyzed. HPAEC-PAD was employed to identify and quantify the COS fraction. All the reactions were carried out under the same conditions and treated in the same way for their comparison.

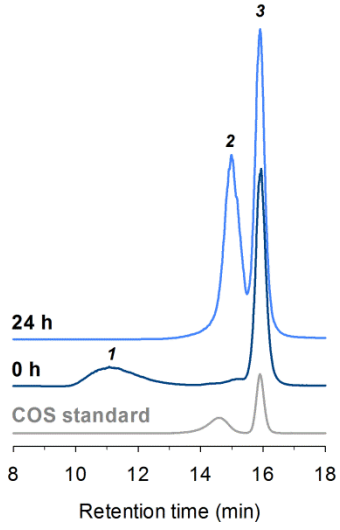


Fig. 3.14. SEC-HPLC analysis of chitosan hydrolysis by Neutrase 0.8L, at 0 h and 24 h. Reaction conditions: 1% (w/v) chitosan CHIT600, 20% (v/v) enzyme, 50 mM sodium acetate buffer pH 5.0, 50°C. Peaks: (**1**) High-molecular-weight chitosan; (**2**) Chitosan oligosaccharides; (**3**) GlcN, GlcNAc and buffer salts. The chromatogram of a COS standard (MW ≤ 2000, DD ≥ 90%) is also shown.

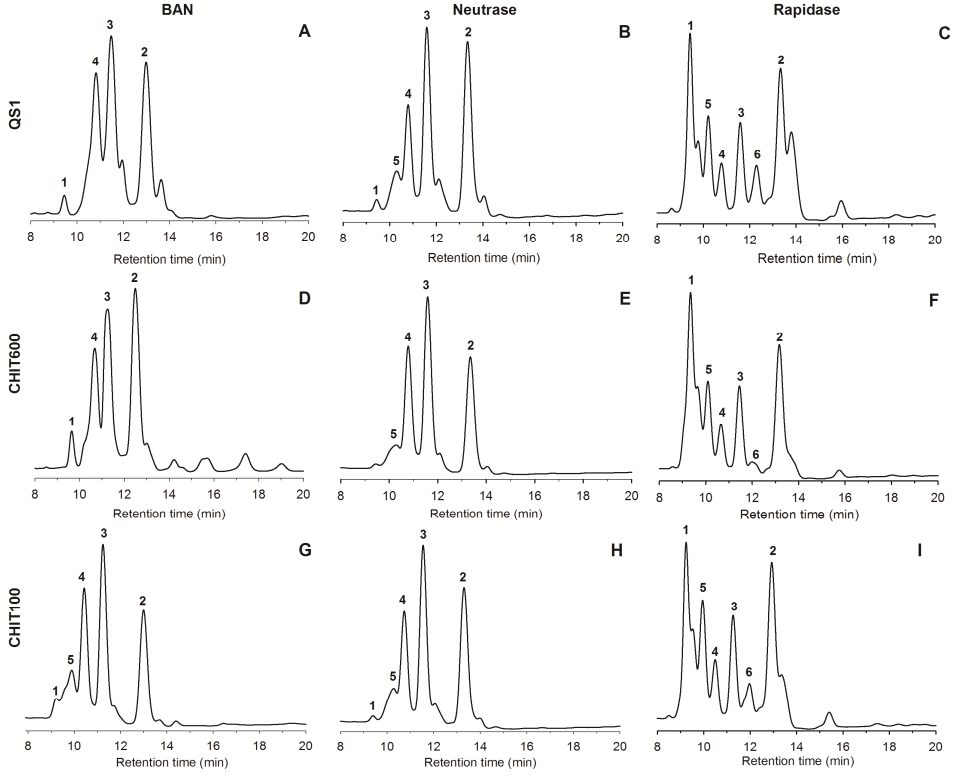


Fig. 3.15. HPAEC-PAD chromatograms of the chitooligosaccharides produced by BAN, Neutrase and Rapidase with chitosans QS1, CHIT100 and CHIT600 as substrates. Reaction conditions: 1% (w/v) chitosan, 20% (v/v) enzyme, 50°C, 50 mM sodium acetate buffer pH 5.0, 24 h. HMW chitosans were precipitated prior to the analysis. Identified peaks: (**1**) GlcN; (**2**) $(GlcN)_2$; (**3**) $(GlcN)_3$; (**4**) $(GlcN)_4$; (**5**) $(GlcN)_5$; (**6**) GlcNAc.

Fig. 3.15 shows the chromatograms at final reaction times (24 h) of the three enzymes and three chitosans (QS1, CHIT600 and CHIT100). Considering the deacetylated nature of the substrate, it is not surprising the absence of fully acetylated COS (faCOS) in the reaction mixtures. On the other hand, the fully deacetylated series (fdCOS) was found in every reaction. However, the three enzymes showed different COS production.

In the case of BAN, the enzyme yielded small amounts of GlcN (peak 1) –that could also be longer COS that coelute at the same retention time (see Chapter 2.4.2)- pointing the activity more towards the endo-type. Neutrase also showed an endo-behavior but with a slight difference: in the case of BAN, there are some unidentified peaks –specially with CHIT600- that would most certainly correspond with partially acetylated COS (paCOS) for which there is no commercial standards; regarding Neutrase, these peaks do not appear, suggesting the presence of a deacetylating activity in the commercial preparation. These results are in agreement with those described for a commercial neutral protease from *B. subtilis* (Li et al., 2005, Li et al., 2007) where the authors postulated that the origin of both activities could be the protease itself. In our case, we have discarded that the chitosanolytic activity came from the protease with the use of activity gels, but strongly support the hypothesis that deacetylation is carried about by the protease, due to the chemical similarity between peptide bonds and N-acetyl bonds. Both Neutrase and BAN are enzymes from the same microorganism, *Bacillus amyloliquefaciens*. Hypothetically, the enzyme responsible for the chitosanolytic activity could be the same. The differences in the acetylation pattern of the products would be a consequence of the deacetylase activity, attributed to the protease, which is not present in the BAN preparation.

Regarding the reaction with Rapidase, it is worth noting the presence of more peaks, with especial interest in peak 6, identified by standards as GlcNAc. The production of both monomers GlcN and GlcNA in the reaction mixtures with Rapidase TF suggests the presence of exo-β-glucosaminidase and/or exo-β-N-acetylglucosaminidase activities in such preparation. The rest of the unidentified peaks would be paCOS, likewise the case of BAN.

Chitosans CHIT600 and CHIT100 present the same DD but different size. These results indicated that the enzymes reached the same hydrolysis degree regardless the molecular weight of the chitosan employed as substrate. On the other hand, the DD of the chitosan and the acetylation pattern exerted a notable influence on the reaction product profile, with the more acetylated chitosan, QS1, yielding a higher amount of paCOS.

3.3.4 Identification of COS by mass spectrometry

Mass spectrometry (ESI-Q-TOF) was very useful to identify (or to discard) the presence of certain COS in the reaction mixtures. Several COS and paCOS were identified based on $[M+H]^+$ and $[M+Na]^+$ signals. The different mass spectra can be found in Appendix I (**Figs. A2.3-11**) and **Fig. 3.16** shows a representative spectrum from the reaction of Rapidase with CHIT600 as substrate.

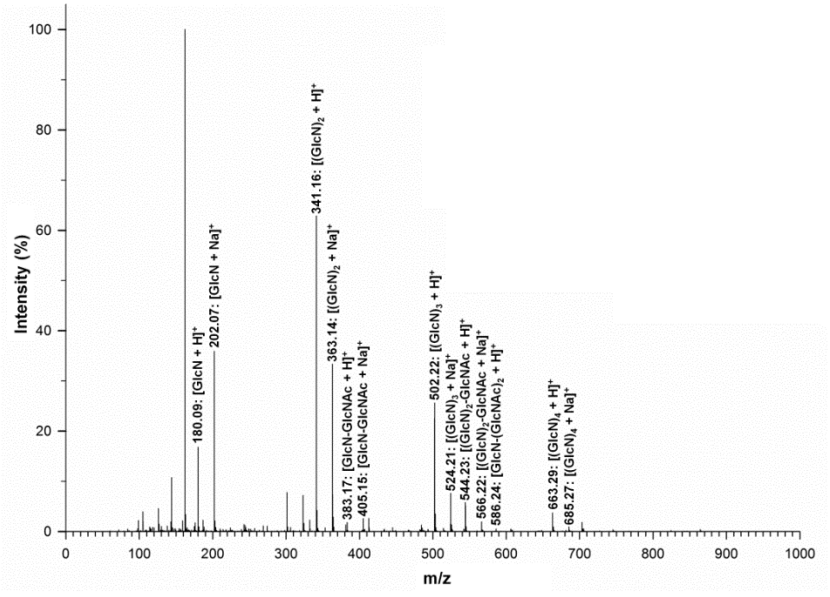
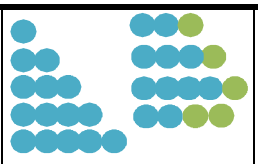
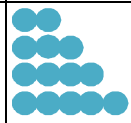
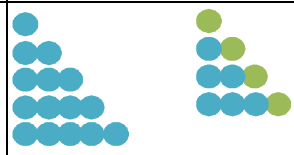
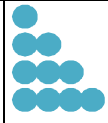
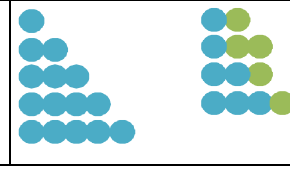


Fig. 3.16. ESI-TOF mass spectrum of the reaction of chitosan CHIT600 with Rapidase TF. Reaction conditions: 1% (w/v) CHIT600, 20% (v/v) enzyme, 50°C, pH 5.0, 24 h.

MS spectra confirmed that Neutrase 0.8L only formed deacetylated products, which supports the hypothesis of the presence of a deacetylating enzyme in this preparation as was deduced from the HPAEC-PAD chromatograms. Even in the case of the chitosan with lower DD (QS1, 81%), all the identified products were fdCOS, except for a minor contribution of $(GlcN)_2$-GlcNAc (**Table 3.5**).

Table 3.5. Mass spectrometry analysis. Schematic representation of polymerization degree and composition of reaction products, using the different chitosans and enzymes.

Chitosan	BAN	Neutrase	Rapidase
QS1 (98 kDa, DD 81%)			
CHIT100 (100-300 kDa, DD > 90%)			
CHIT600 (600-800 kDa, DD > 90%)			

● Glucosamine (GlcN); ● N-acetyl-glucosamine (GlcNAc)

On the contrary, Rapidase TF and BAN yielded a mixture of fdCOS and paCOS, with the difference of the lack of GlcNAc in the BAN mixtures. It should be emphasized that COS with the same composition but arranged in different order gave only one signal in the mass spectrum; for that reason, the reaction mixtures are probably even more complex than can be inferred from the MS analysis.

3.3.5 Quantification of identified COS by HPAEC-PAD

Once the identity of the peaks was confirmed with MS-ESI, calibration curves were built in the HPAEC-PAD method for the confirmed COS. Considering total conversion of chitosan in every case, as deduced from SEC-HPLC, the concentration of paCOS was estimated as the difference between the sum of known COS and the initial concentration of chitosan (10 g/L). However, some of the peaks might be co-eluting with some of the identified COS. For that reason, one may be cautious with the presented values, but they give us an idea of the proportion of COS in the final reaction mixtures. Only in the case of Neutrase an overall yield of COS production could be calculated, thanks to its deacetylating activity that facilitated the identification of all the products. Neutrase, after 24 h reaction with CHIT600 or CHIT100 as substrates, synthesized approximately 2.5 g/L of $(GlcN)_2$, 4.5 g/L of $(GlcN)_3$ and 3 g/L of $(GlcN)_4$ (yield 100%) (**Fig. 3.17**).

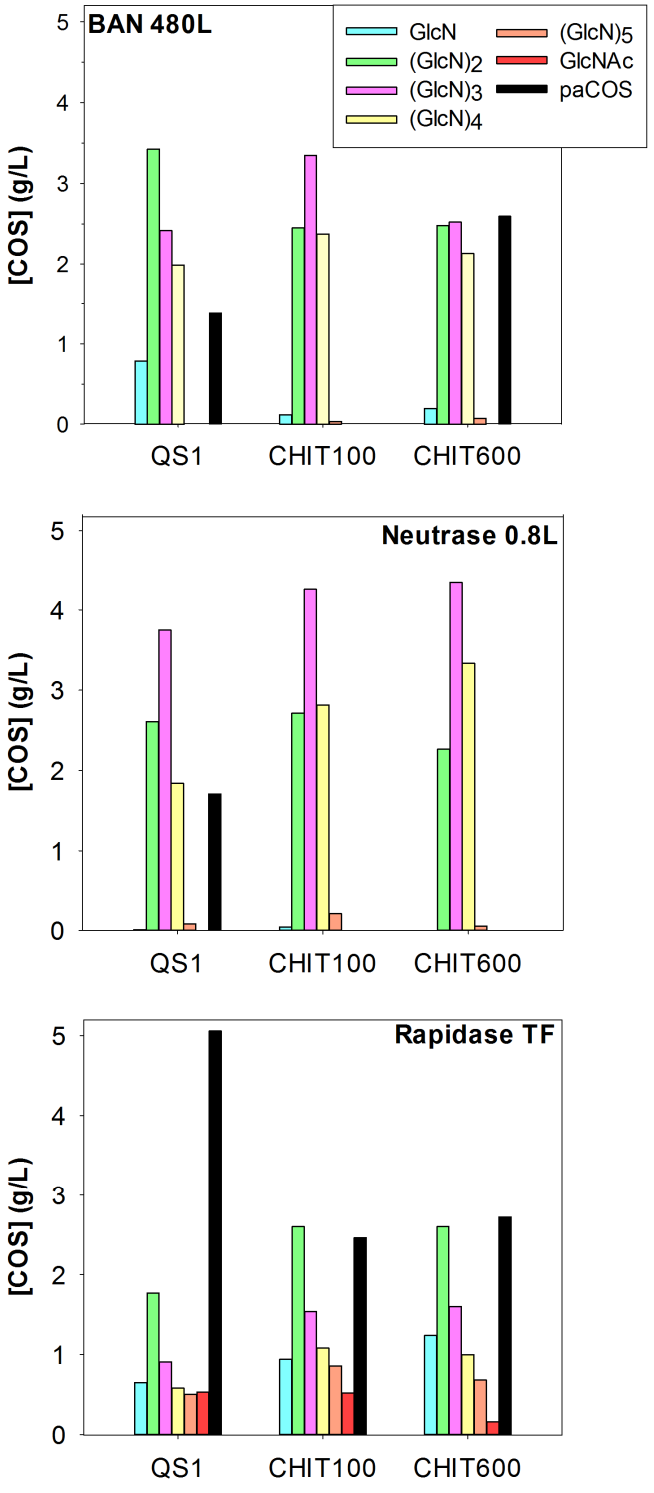


Fig. 3.17. Quantification of identified COS by HPAEC-PAD. Reaction conditions: 1% chitosan (w/v), 20% (v/v) enzyme, 50°C, 50 mM sodium acetate buffer pH 5.0, 24 h.

3.4 Reactors for the production of chitooligosaccharides

3.4.1 Micro-scale immobilization screening

Considering the large molecular size of chitosan, the immobilization of the chitosanolytic activity should preferably take place on the surface of the carrier, either by adsorption or, more desirable, by the formation of covalent bonds. In fact, the literature shows that most of the methods previously used for the immobilization of chitosanolytic enzymes are covalent (see Appendix I, **Table A4.1**).

The immobilization method used earlier in this Thesis for FOS synthesis, covalent immobilization on VS-activated silica carriers (Chapter 3.2), was tested with these enzymes but it was not successful (data not shown). As an alternative, previous work in our laboratory with other enzymes had proved that glyoxal agarose carriers had a good performance in packed-bed reactors (Rodriguez-Colinas et al., 2016) and were therefore assayed.

The three chitosanolytic enzymes described in Chapter 3.3 were immobilized following the micro-scale procedure described in Chapter 2.7. Apparent activities of the biocatalyst and total yields are summarized in **Table 3.6.** Yields in terms of activity were only possible to be calculated for BAN, due to some instability of Neutrase and Rapidase at the immobilization conditions (pH 10.0).

Table 3.6. Micro-scale immobilization screening of chitosanolytic enzymes

		Theoretical[b]		Apparent[c]	
Biocatalyst	Initial[a] (U)	Activity (U/g)	Yield (%)	Activity (U/g)	Yield (%)
BAN 480 L	0.7 ± 0.09	5.4 ± 0.5	41.54 ± 5.3	2.91 ± 0.04	24.3 ± 0.5
Neutrase 0.8L	n.d.	n.d.	n.d.	0.92 ± 0.11	n.d.
Rapidase TF	n.d.	n.d.	n.d.	0.06 ± 0.01	n.d.

[a] Measured in a control of soluble enzyme after incubation at the immobilization conditions (pH 10).
[b] Theoretical values determined by subtracting the total initial activity prior to immobilization and the remaining activity in the filtrate and washing solutions after immobilization.
[c] Experimental activity (standard assay).
n.d. not determined due to instability issues.

BAN showed the highest activity, close to 3 U/g. Therefore, it was selected for its immobilization and the design of processes for the production of COS in continuous bioreactors.

3.4.2 Covalent immobilization of BAN 480L

BAN 480L was covalently coupled to glyoxal agarose beads, yielding the biocatalyst BAN-Glx (**Fig. 3.18**). The recovery of activity was not very high (24%, **Table 3.6**) probably due to the typical considerations involving covalent immobilization. First, covalent immobilization involves the rigidification of the enzyme structure causing some loss of activity. Secondly, the binding can take place in an orientation that hides the active site of the enzyme not allowing the diffusion of chitosan. Finally, the enzyme might show some instability at the immobilization pH (10.0). However, the activity (3 U/g) showed by the biocatalyst BAN-Glx was considered high enough to set up a bioreactor. The immobilization was scaled to 1 gram of biocatalyst in order to obtain more immobilized enzyme for further experiments; the yield and apparent activity were maintained.

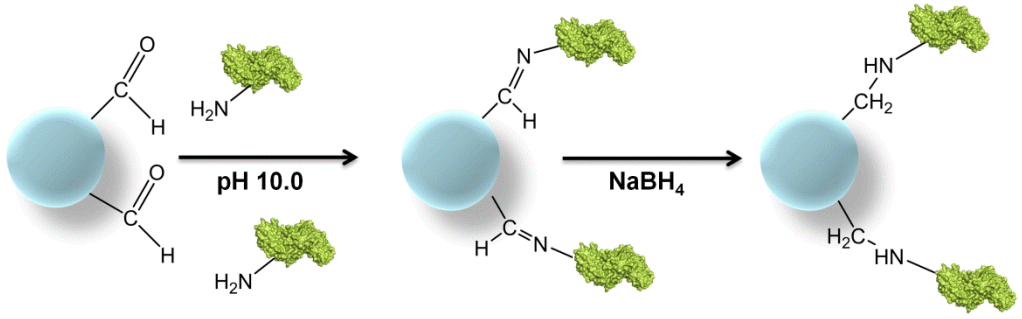


Fig. 3.18. Enzyme immobilization mechanism on glyoxal agarose beads.

3.4.3 Characterization of BAN-Glx

The effect of immobilization on enzyme stability under reaction conditions was assayed and compared with the soluble enzyme (**Fig. 3.19**). BAN-Glx was substantially more stable at 50ºC (pH 5.0) than the soluble enzyme. BAN-Glx conserved around 60% of its initial activity after 2 days under such conditions. This stabilization effect is typically reported in covalent immobilization (Eş et al., 2015).

The production of COS by BAN-Glx was analyzed by HPAEC-PAD and MS-ESI, and compared with the soluble enzyme. Reactions were performed with 0.5% (w/v) chitosan CHIT100 and the same amount of enzyme units (0.05 U/mL), in a total reaction volume of 3 mL, and were followed during 4 h. More diluted substrate solutions, in comparison with previous experiments, were employed to avoid viscosity problems that lead to poor mixing with the immobilized biocatalyst. **Fig. 3.20** shows the progress of formation of the identified fdCOS with soluble and immobilized BAN. The reaction profiles with free and immobilized biocatalysts were very similar. In both cases, $(GlcN)_5$ was initially formed and further hydrolyzed to $(GlcN)_2$ and $(GlcN)_3$. MS-ESI spectra (Appendix I, **Figs. 2A12-14**) obtained with the three chitosans and BAN-Glx were very similar to the obtained with the soluble enzyme (**Table 3.5**).

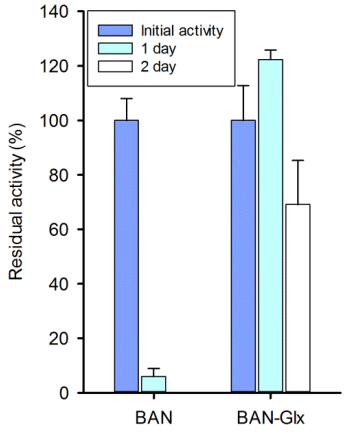


Fig. 3.19. Enzyme stability at the optimal reaction conditions. BAN and BAN-Glx were incubated at 50°C in 50 mM acetate buffer (pH 5.0). Relative activity is referred to the activity previous to any incubation.

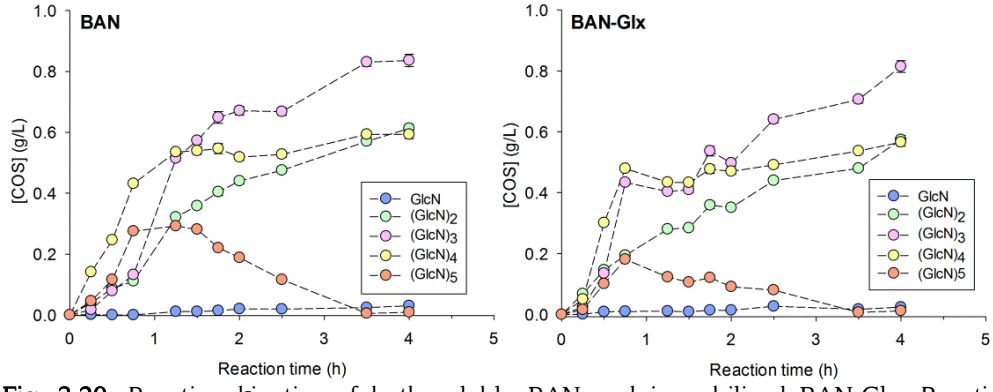


Fig. 3.20. Reaction kinetics of both soluble BAN and immobilized BAN-Glx. Reaction conditions: 0.5% (w/v) chitosan CHIT100, 0.05 U/mL enzyme, 50°C, 900 rpm.

In general terms, the immobilization did not change the COS formation kinetics and the product profile, but significantly increased the stability of the enzyme.

3.4.4 Batch reactor with BAN-Glx and rheology of chitosan

A 100-mL batch stirred tank reactor was set up as described in Chapter 2.10. Viscosity and COS concentration were monitored for 60 h (**Fig. 3.21**). Viscosity, related with the presence of HMW chitosan, decreased very fast without significant appearance of COS in the reaction (**Fig. 3.21**). This fact points out the specificity of the enzyme, which endo-hydrolyzes the chitosan and therefore decreases the size of the substrate very fast, without the appearance of COS oligomers (detected by HPAEC-PAD). Overall productivity of this batch reactor was 1.7 g_{COS} L^{-1} h^{-1}, which is not particularly high compared with the productivities that can be achieved operating in continuous mode (Jeon and Kim, 2000, Kuroiwa et al., 2003, Kuo et al., 2004).

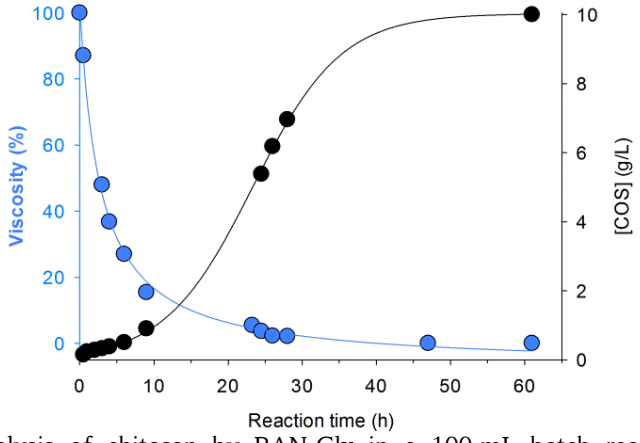


Fig. 3.21. Hydrolysis of chitosan by BAN-Glx in a 100-mL batch reactor. (Blue axis) Changes in viscosity with reaction time. (Black axis) Formation of total COS with reaction time. Reaction conditions: 1% (w/v) CHIT100 in 100 mM acetate buffer (pH 5.0), 0.015 U/mL. The reactor worked at 50ºC and 400 rpm, with magnetic stirring.

3.4.5 Packed-bed reactor with BAN-Glx

In order to increase productivity, BAN-Glx was packed in a 1 mL glass column for the continuous production of COS. 1% (w/v) chitosan (CHIT100) was pumped through the PBR at 0.1 mL/min. Unfortunately, due to the high viscosity of chitosan and the compressible nature of the agarose beads, column clogging and further reduction of the flow rate took place, leading finally to reactor shutdown. Dilution of the feed chitosan solution to 0.5% (w/v) did not improve the flow.

Based on previous reports by Rakmai and coworkers (Rakmai and Cheirsilp, 2016) for the synthesis of β-cyclodextrins from starch slurry, we considered a two-step process, in which chitosan was first hydrolyzed in a CSTR to a viscosity that could flow through the PBR (**Fig. 3.22**).

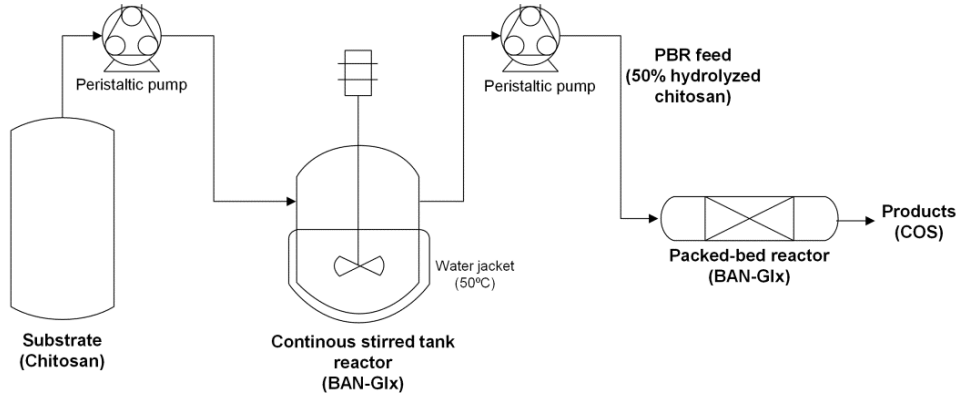


Fig. 3.22. Dual reactor system. Flow diagram of a dual reactor with a CSTR to partially hydrolyze chitosan and a PBR fed with 50% partially hydrolyzed chitosan for the complete transformation of chitosan into COS.

In order to do so, the critical viscosity that the system could accept was assessed. Theoretical modelling of this specific system is not very straightforward due to two main factors: the non-Newtonian nature of chitosan, which makes it difficult to know the exact value of viscosity in a PBR (Park et al., 1975); and the compressibility of the carrier, which adds another factor to the modelling (Stickel and Fotopoulos, 2001). Moreover, the agarose carriers swell in aqueous solution, complicating the measurements of particle size and voidage of the column. Taking all this into account, we decided to follow an empirical approach. Different batch reactors were set-up with the immobilized enzyme and reactions were stopped at different degrees of hydrolysis, measured by the reduction in viscosity. Completely hydrolyzed chitosan (defined as degree of hydrolysis of 100%) was assessed, and likewise hydrolysis levels of 90, 85, 55, and 40% were tested. The partially hydrolyzed chitosan was pumped at 0.2 mL/min through the PBR. Column clogged after 2 h with 40% hydrolyzed chitosan, indicating that the viscosity of this substrate was over the critical value that the column could accept. In contrast, 50% hydrolyzed chitosan did not cause any clogging and was therefore used for the PBR experiments.

The effect of dilution rate (d, h^{-1}) on productivity and overall yield was studied (**Fig. 3.23**) with 1% (w/v) CHIT100 (50% hydrolyzed) as substrate ($[Chitosan]_o$), using the following equations:

$$\text{Conversion yield (\%)} = \frac{[COS]}{[Chitosan]_0} \times 100 \quad (1)$$

$$\text{Productivity } (g_{COS}\ L^{-1}h^{-1}) = [COS] \times d \quad (2)$$

At the lowest dilution rate the productivity was 37 g_{COS} L^{-1} h^{-1}, with a conversion yield of 73%. On the other hand, the highest dilution rate (corresponding to a flow rate of 1 mL/min) gave a productivity of nearly 200 g_{COS} L^{-1} h^{-1}, but at the cost of the conversion yield, which dropped to around 40%, as a result of shorter residence time. Regarding product selectivity, the mixture presented a higher content of longer chitooligosaccharides when increasing the dilution rate. A total conversion of chitosan into COS would be desirable in order to eliminate the purification step to remove the residual chitosan. It would be interesting to reduce the dilution rate to the maximum allowable to avoid the use of downstream processes of purification. Nevertheless the principle of the dual-reactor seems to work well and productivity and yield obtained in this work are higher than those described in the literature for the continuous production of COS in the PBR alone (Ichikawa et al., 2002, Kuroiwa et al., 2003).

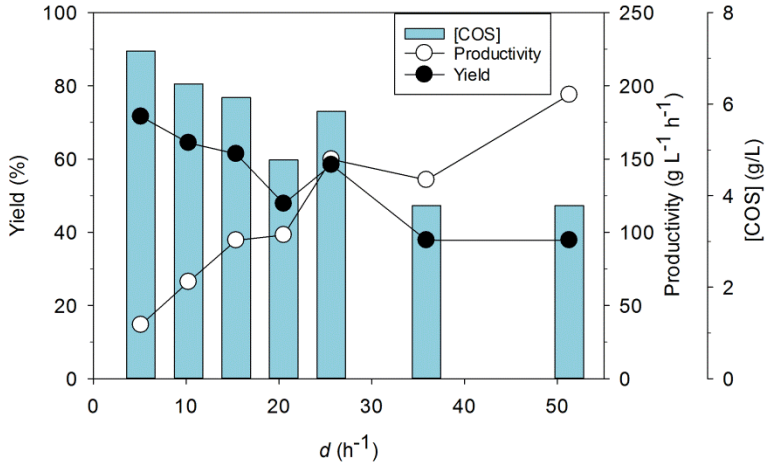


Fig. 3.23. Effect of dilution rate on the operation of the PBR. Changes in COS concentration (bars), productivity (white circles), and conversion yield (black circles) were measured at different flow rates.

3.5 Novel sources of chitosanolytic activity

Even though the chitosanolytic activity in the commercial enzymes was acceptable, we decided to look for more specific activities, in order to exert a higher control on the composition of the final product.

3.5.1 Chitosanolytic activity in *Bacillus thuringiensis*

As described in the Introduction of this Thesis, several chitosanases have been described in *Bacillus* strains. In this context, *Bacillus thuringiensis* is a soil spore-forming bacterium widely employed as biopesticide in agriculture and forestry as it synthesizes crystal proteins that are toxic to many species of insects, but not to humans (Barboza-Corona et al., 2012). This bacterium also displays a series of enzyme activities such as proteinase, chitinase (Barboza-Corona et al., 2008) and chitosanase (Chen et al., 2010), which are probably involved in its insecticidal and antifungal action, and could expand the perspectives of application of this microorganism. In particular, Cruz-Camarillo and cols. first described the existence of chitosanase activity in various strains of *B. thuringiensis* (Cruz-Camarillo et al., 2004). Also, Lee and cols. reported the isolation of several of these enzymes (Lee et al., 2007). *B. thuringiensis* chitosanases belong to the GH8 family of glycoside hydrolases and exhibit high sequence identity (>96%) to other chitosanases from different *Bacillus* species. Although some of these enzymes have been biochemically characterized, their full potential for the production of chitooligosaccharides remains unexplored.

Two commercial preparations of *Bacillus thuringiensis* (var. *aizawai* and var. *kurstaki*) widely used as biopesticides (XenTari® and Belthrul®, respectively) were selected as potential sources of chitosanolytic activity. Optical microscopy of the aqueous suspensions (10 mg/mL) showed the presence of the typical cells and spores of this bacterium (Appendix I. **Fig. A3.2**) (Melo et al., 2016).

Only the cells from var. *aizawai* displayed significant activity towards chitosan by the DNS method. Interestingly, an aqueous wash was not able to extract the chitosanolytic activity present in this bacterium, which indicated that the enzyme was probably tightly associated to the cell surface. We assayed different

extracting agents to solubilize the enzyme, and finally we found that 1 M sodium acetate extracted approximately 95% of the initial activity in the case of the XenTari® preparation (**Table 3.7**). Starting with 4 g cells, we obtained 2.9 mL of an enzyme solution (Bt-chitosanase) showing 5.1 U/mL, with chitosan CHIT600 as substrate. This activity was higher than the measured in the commercial preparations described earlier in this Chapter (**Table 3.4**).

Table 3.7. Extraction of chitosanolytic activity of *B. thuringiensis* var. *aizawai* with 1 M sodium acetate.

Initial (g)	$V_{initial}$ (mL)	V_{final} (mL)	[Protein] (mg/mL)	Activity	
				Volumetric[a] (U/mL)	Yield[b] (%)
4	50	2.9	0.26	5.1	95%

[a] Measured by the DNS method

[b] Referred to the initial activity in the cells

The effect of temperature and pH towards activity and stability of Bt-chitosanase preparation was studied in order to establish the optimal conditions for the synthetic reaction. **Fig. 3.24A** shows that maximum activity was found at pH 6.0. It was not possible to assay the activity at higher pH values due to the limited solubility of chitosan at pH > 6 and the difficulty to prepare such solutions. According to previous studies using colloidal chitosan (Cruz-Camarillo et al., 2004), optimum pH is close to 7.0 for chitosanases from *B. thuringiensis* strains. The enzyme was significantly stable at pH values between 5.0 and 7.0. Chen at al. (2010) also observed maximum pH stability around pH 7 for a chitosanase from *B. thuringiensis* (Chen et al., 2010). A pH value of 5.0 was selected for the controlled hydrolysis of chitosan into chitooligosaccharides, as it represented a compromise between maximum enzyme activity/stability and chitosan solubility. Regarding the effect of temperature (**Fig. 3.24B**), maximum activity was obtained at 60°C, in accordance with previous studies of chitosanolytic activity of *B. thuringiensis* (Lee et al., 2007). The thermostability of Bt-chitosanase in acetate buffer (pH 5.0) was optimum at 40°C but quite low at 60°C; such thermolability was also described for the chitosanase from *B. thuringiensis* ZJOU-010 (Chen et al., 2010) and for most of the reported chitosanases (Thadathil and Velappan, 2014). However, in presence of 1% (w/v) chitosan the thermostability substantially increased, and the enzyme

remained active at 60°C during at least 48 h. At this temperature the viscosity of chitosan solution is lower and the reaction rate can be increased, also minimizing microbial contamination. The stabilizing effect of carbohydrates has been previously reported with several glycosidic enzymes (Prasad and Roy, 2010, Ghazi et al., 2007, Rodriguez-Colinas et al., 2011) including chitosanases (Cruz-Camarillo et al., 2004, Somashekar and Joseph, 1996).

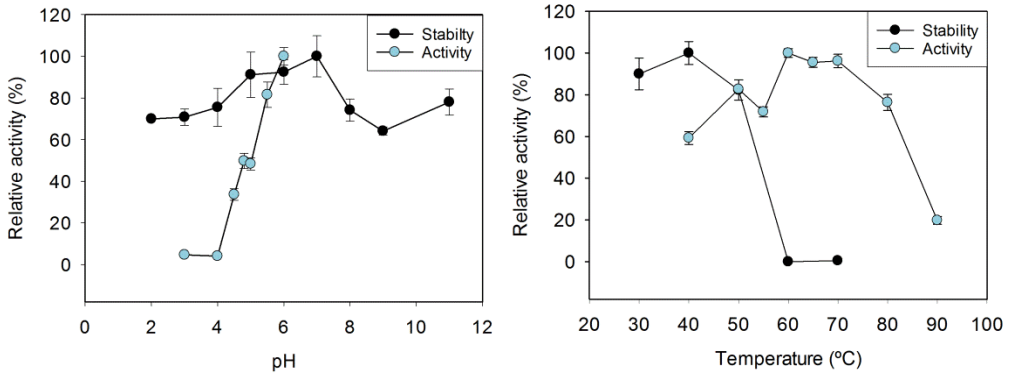


Fig. 3.24. Effect of pH (**A**) and temperature (**B**) on activity (blue circles) and stability (black circles) of Bt-chitosanase. Relative activity is referred to the initial activity of the enzyme, prior to any incubation, using the standard activity assay.

The hydrolysis of 1% (w/v) chitosan with Bt-chitosanase was carried out in 0.05 M sodium acetate buffer (pH 5) at 60°C. Reaction rate was notably faster with the more deacetylated chitosan (CHIT600), indicating that the activity extracted in *B. thuringiensis* was more chitosanolytic than chitinolytic (Chen et al., 2010).

The chitooligosaccharide fraction obtained by the hydrolysis of both chitosans was analyzed by HPAEC-PAD and MS-ESI as described before. In the case of CHIT600 (**Fig. 3.25**, black chromatogram), four major peaks were identified, corresponding to the deacetylated products chitobiose $(GlcN)_2$, chitotriose $(GlcN)_3$, chitotetraose $(GlcN)_4$ and chitopentaose $(GlcN)_5$. With the chitosan QS1 (blue chromatogram) the main difference was the presence of more unidentified compounds, especially at longer retention times, which probably corresponded to paCOS in accordance with their higher degree of acetylation. The reaction products were very similar to those obtained by commercial enzyme BAN 480L (**Fig. 3.15**, **Table 3.6**), indicating the action of an endo-chitosanolytic enzyme.

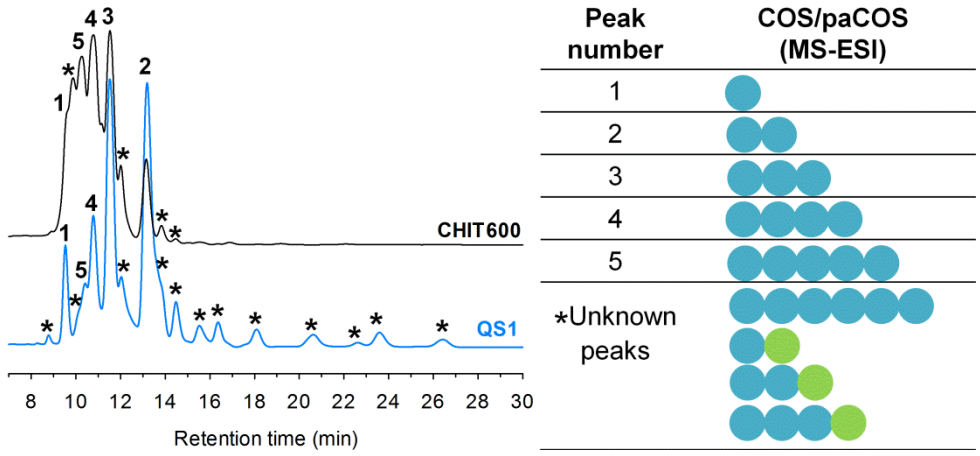


Fig. 3.25. (**Left**) HPAEC-PAD chromatograms of the chitooligosaccharides produced by Bt-chitosanase using chitosans CHIT600 (24 h, black chromatogram) and QS1 (88 h, blue chromatogram) as substrates. Reaction conditions: 1% (w/v) chitosan in 50 mM sodium acetate buffer pH 5.0, 60°C, 0.05 U/mL. Identified peaks: (**1**) GlcN + COS with DP ≥ 7; (**2**) $(GlcN)_2$; (**3**) $(GlcN)_3$; (**4**) $(GlcN)_4$; (**5**) $(GlcN)_5$. (**Right**) Schematic representation of fdCOS and paCOS detected by MS-ESI, related with the peak assignation in the HPAEC-PAD chromatogram. Blue circles: GlcN. Green circles: GlcNAc.

Several fdCOS and paCOS were identified based on $[M+H]^+$ and $[M+Na]^+$ signals (Appendix I, **Figs. A2.15-16**). Apart from the $(GlcN)_n$ (n=1-5) already identified by HPAEC-PAD, MS revealed the presence of $(GlcN)_6$, which would probably correspond to the peak on the left of $(GlcN)_5$ peak in the chromatogram (**Fig. 3.25**). The MS spectra also showed the presence of several paCOS containing one residue of GlcNAc: GlcN-GlcNAc, $(GlcN)_2$-GlcNAc and $(GlcN)_3$-GlcNAc. Such compounds (and their isomers) probably corresponded to some of the minor peaks not identified in the HPAEC-PAD chromatograms (**Fig. 3.25**).

The reaction of chitosan CHIT600 with Bt-chitosanase was followed during 72 h (**Fig. 3.26**). Chitosan was fully converted into oligosaccharides in approximately 55 h, using 0.05 U/mL of enzyme. The main synthesized product was $(GlcN)_4$. The final composition at the equilibrium was as follows: 1.6 g/L of $(GlcN)_2$, 1.7 g/L of $(GlcN)_3$, 5 g/L of $(GlcN)_4$ and 1.4 g/L of $(GlcN)_5$. A minor amount of GlcN (0.04 g/L) was also detected. The rest of carbohydrates in the reaction mixture (approx. 0.3 g/L) were paCOS and longer fdCOS. Using normal-phase HPLC to separate the chitosan oligomers, Lee et al. (2007) reported that the main products synthesized by several chitosanases from other *B. thuringiensis* strains were chitotriose and

chitotetraose (Lee et al., 2007). However, the chitosanases from other *Bacillus* species (*B. subtilis, B. circulans)* yielded the dimer (chitobiose) as the major product followed by the trimer (chitotriose) (Chiang et al., 2003, Fukamizo et al., 2005). Recently, a recombinant chitosanase from *Bacillus* sp. formed $(GlcN)_4$ and $(GlcN)_5$ as the main oligosaccharides (Zhou et al., 2015).

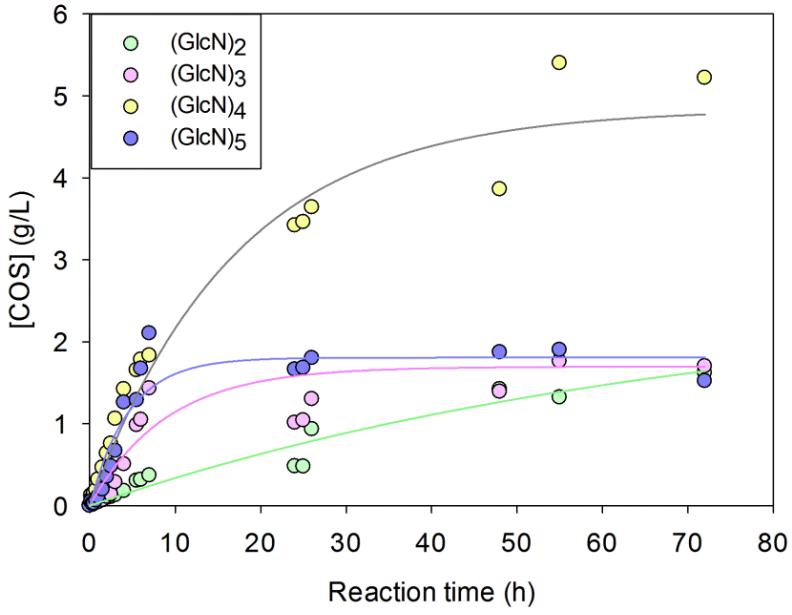


Fig. 3.26. Evolution of COS production with chitosan CHIT600 and Bt-chitosanase. Only concentrations of the identified products are shown. Reaction conditions: 1% (w/v) chitosan in 50 mM sodium acetate buffer pH 5.0, 60°C, 0.05 U/mL.

3.5.2 Production of COS by chitinases from *Trichoderma harzianum*

The enzymes used for the production of COS in the previous chapters were obtained from available and cost-effective commercial sources. However, the mixture of COS produced, though with slight differences, belonged to the same family: fully deacetylated COS, with a small proportion of partially acetylated COS. In order to obtain mixtures enriched in paCOS and free from fdCOS, the use of chitinases that only hydrolyse bonds involving GlcNAc residues is preferred. If the substrate presents an acetylation degree high enough to offer a significant amount of cutting sites to the enzyme, but not too high to yield only chitin-oligosaccharides (N-acetylated COS, faCOS), the mixture could be enriched in paCOS.

Two chitinases (CHIT33 and CHIT42) from the fungus *Trichoderma harzianum*, isolated and cloned in *Pichia pastorus* in the laboratory of Dr. Maria Fernández-Lobato (CBM, UAM-CSIC) in the context of the collaborative project GLICOENZ (http://www.glicoenz.org/), were used for the experiments. Reactions were analysed with HPAEC-PAD and MALDI-TOF and the chromatograms are shown in **Fig. 3.27**. When assaying chitin as substrate (blue chromatogram), the main products were faCOS, including the monomer GlcNAc. However, there were differences between the two chitinases: CHIT42 yielded mainly $(GlcNAc)_2$, with small amounts of the monomer and trimer, whereas CHIT33 yielded mainly $(GlcNAc)_4$, followed by the dimer, the trimer, and also the monomer. These results are in agreement with the reactions catalysed by the native enzymes (De La Cruz et al., 1992). Even though both chitinases belong to the GH18 family (CAZy database), the differences in the production of faCOS might be related with processivity, which is the ability of the enzymes to catalyse consecutive reactions without releasing the substrate. In the case of CHIT42 the enzyme seems to have this property, binding the substrate and catalysing the hydrolysis every two residues and therefore yielding mainly the dimer. In the case of CHIT33 this does not seem to be the case, therefore forming different faCOS and not only the dimer.

The catalytic mechanism of the chitinases of the GH18 family requires the presence of GlcNAc in the –1 position (Boer et al., 2004, Arakane et al., 2012). This explains the production of only paCOS when chitosan is used as substrate. Because chitosan is mostly deacetylated, low yields could be explained by large zones of substrate lacking GlcNAc residues that the enzyme cannot hydrolyse.

Reactions using chitosan as substrate (**Fig. 3.27**, black chromatograms) yielded only two identifiable peaks by HPAEC-PAD: GlcNAc and N,N'-diacetylchitobiose (peaks 6 and 7, respectively). The rest of the peaks did not elute at any known retention time for either acetylated or deacetylated COS, meaning they were most probably paCOS, as was confirmed by mass spectrometry. The technique used in this case for the MS analysis was MALDI instead of ESI, because the expected products were longer than with the chitosanases. **Fig. 3.28** and **Table 3.9** show the results obtained for CHIT42. Unfortunately, CHIT33 reaction mixture did not ionize

well for MALDI, and it was not possible to analyse its reactions this way. However, the profile of COS expected is more or less the same as with CHIT42, as was confirmed by the products that could be detected by MS/ESI (Appendix I, **Fig. A217**).

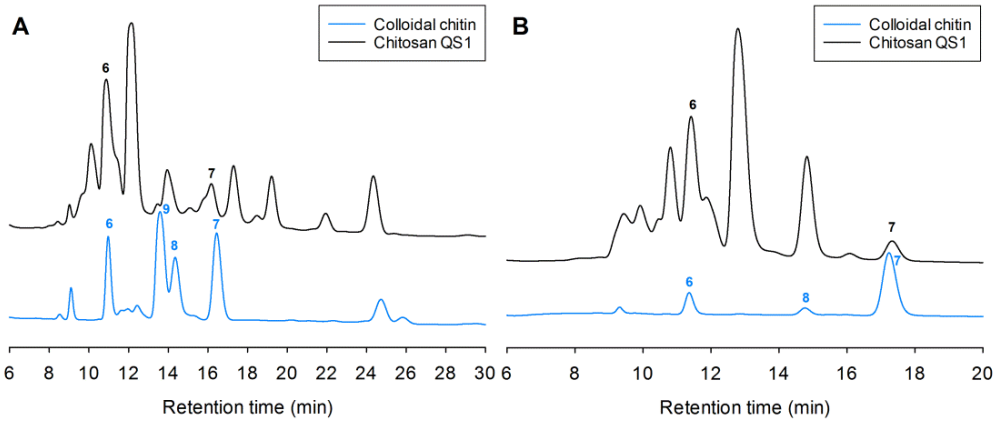


Fig. 3.27. HPAEC-PAD chromatograms of CHIT33 (**A**) and CHIT42 (**B**) reactions with colloidal chitin (blue) and chitosan QS1 (black) as substrate. Reaction conditions: 1% substrate, 10% (v/v) enzyme, 50 mM sodium acetate buffer pH 5.5, 35°C. Peaks: (**6**) GlcNAc; (**7**) $(GlcNAc)_2$; (**8**) $(GlcNAc)_3$; (**9**) $(GlcNAc)_4$.

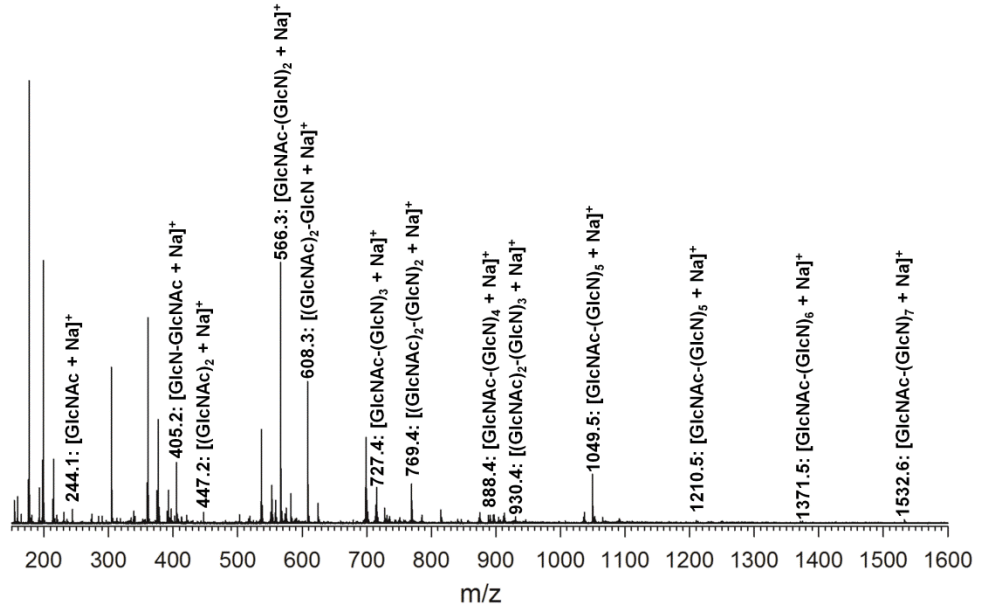
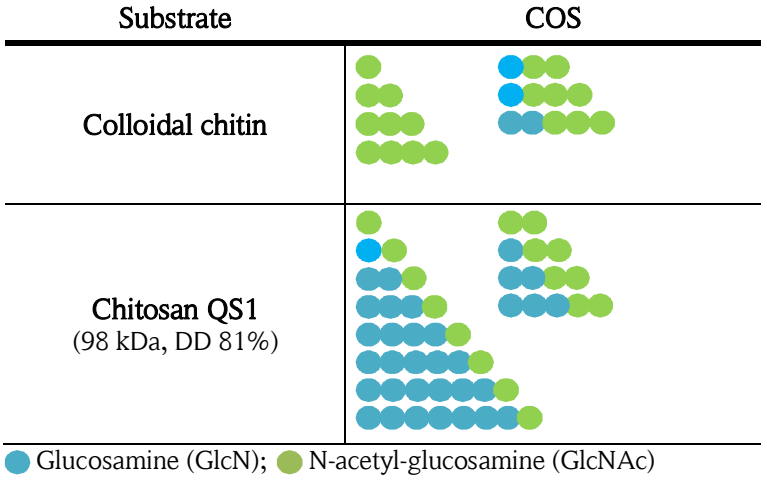


Fig. 3.28. MALDI-TOF spectrum of the reaction of chitosan QS1 with CHIT42. Reaction conditions: 1% (w/v) QS1, 20% (v/v) CHIT42, 35°C, pH 5.5, 24 h. The peaks corresponding to identified COS are indicated.

Table 3.8. MALDI-TOF mass spectrometry analysis. Schematic representation of polymerization degree and composition of reaction products using chitinase CHIT42 as biocatalyst.



MALDI experiments confirmed the presence of faCOS in the reaction mixture with colloidal chitin as substrate, but there were also some paCOS detected (**Table 3.8**). This is related with the fact that chitin is not 100% acetylated, and can also suffer some deacetylation events in the process of the colloidal chitin preparation. Regarding the products obtained with chitosan as substrate they were all paCOS with at least one GlcNAc residue, presumably in the reducing end.

The biological properties of this kind of mixtures enriched in paCOS have been scarcely studied in comparison with fully acetylated or deacetylated COS. For instance, Park and cols. described the inhibitory effect of Angiotensin I Converting enzyme (ACE), related with hypertension, by a series of paCOS mixtures, but the exact composition of the samples was not analysed (Park et al., 2003b).

The reaction with CHIT42 and chitosan QS1 as substrate was scaled up (20 mL) and paCOS were purified by membrane filtration (cut-off 3 kDa) to eliminate the enzyme and the non-hydrolyzed chitosan. The resulting paCOS (< 3 kDa) were lyophilized and further desiccated. The production yield was under 2%. The non-hydrolysed chitosan from this reaction was used as substrate in a reaction with Rapidase as biocatalyst to obtain a mixture of fdCOS enriched in chitobiose (Appendix I, **Fig. A5.3**). The production yield in this case was close to 100%.

3.7. Antioxidant and neuroprotective activities of COS and FOS

Oxidative stress arises from an excessive formation of reactive oxygen species (ROS). ROS are produced in the forms of H_2O_2, superoxide anion (O_2^-) and hydroxyl ($OH^\cdot$) radicals. Free radicals attack macromolecules such as lipids, proteins, and DNA. In humans this is related with the development of age-related diseases such as atherosclerosis, cancer, or neurodegenerative diseases like Alzheimer's disease (Kerch, 2015). Antioxidants can therefore enhance human health by protecting the body against ROS attack. But antioxidants are also of interest in other fields, for example in the food industry. Stored food can suffer oxidation starting from lipid oxidation, which can influence protein oxidation causing, at the end, deterioration of food quality, rancidity and shortening of shelf life (Ngo and Kim, 2014).

Antioxidant activity of fully deacetylated COS (fdCOS), produced by hydrolysis (and deacetylation) of chitosan CHIT600 by Neutrase, paCOS produced by hydrolysis of chitosan QS1 by chitinase CHIT42 and FOS mixtures were tested by different assays and compared with individual COS, FOS and other known antioxidants.

3.7.1 ABTS radical scavenging activity

Scavenging activity of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical was measured in different mixtures containing COS and FOS: fdCOS produced by Neutrase, fdCOS produced by a combination of CHIT42 and Rapidase, faCOS produced by CHIT42, commercial COS, commercial FOS, and FOS and COS standards (see composition of mixtures in Appendix I, **Figs. A5.2-6**). All compounds were tested at a fixed concentration of 5 g/L for 10 min and scavenging activity was expressed as the reduction in absorbance of ABTS radical, and compared with Trolox (100%), a known antioxidant analogue of tocopherol (**Fig. 3.29**).

Neither the FOS mixtures nor the monomers GlcN and GlcNAc showed any antioxidant activity. The COS mixture containing partially acetylated COS showed the highest scavenging capacity, followed by commercial COS, that are mostly

deacetylated, fdCOS produced by Rapidase-CHIT42 and, lastly, by fdCOS produced by Neutrase. These differences between the fdCOS mixtures can arise from the fact that the Rapidase-CHIT42 mixture contains mainly $(GlcN)_2$ and this disaccharide showed the highest activity compared with the others standards of the deacetylated series. However, the Rapidase-CHIT42 mixture contains a high proportion of the glucosamine monomer, which has shown some cytotoxicity during *in vivo* experiments (de Assis et al., 2012) and lacks antioxidant activity. Considering all the evidence, this mixture was discarded for future experiments.

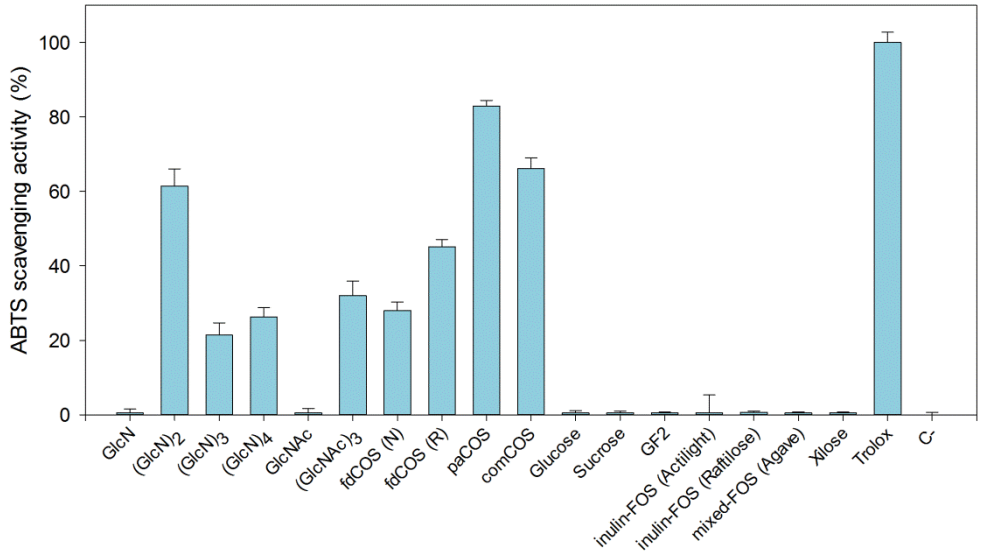


Fig. 3.29. ABTS radical scavenging activity of different COS and FOS mixtures compared with Trolox (100%) and a negative control (0%). fdCOS (N): fdCOS produced by Neutrase. fdCOS (R): fdCOS produced by a combination of CHIT42 and Rapidase. GF2: 1-kestose. C-: negative control (acetate buffer).

The main product in the paCOS mixture is the trimer $(GlcN)_2$-GlcNAc. This fact indicates that the mechanism by which chitooligosaccharides show antioxidant activity requires the combination of both N-acetyl and deacetylated groups in the molecule, as supported by other experiments in the literature (Li et al., 2013).

For those compounds that displayed antioxidant activity and for which we had enough concentration available (COS mixtures and $(GlcN)_2$), the half maximal scavenging concentration (SC_{50}), defined as the concentration in mg/mL at which the compound is able to decrease 50% the absorbance of ABTS radical in ten

minutes, was calculated and is represented in **Fig 3.30** and **Table 3.9.** As expected from **Fig. 3.29**, the best values of SC_{50} are those of the paCOS mixture.

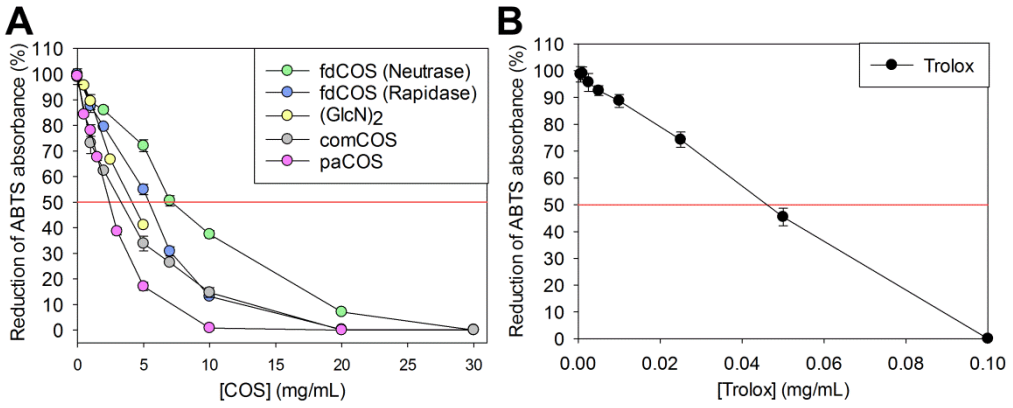


Fig. 3.30. Half maximal scavenging concentration (SC_{50}) of different COS mixtures and standards (**A**) and Trolox (**B**).

Table 3.9. Half maximal scavenging concentration (SC_{50}) of COS mixtures and standards.

	SC_{50} (mg/mL)
fdCOS (N)	7.2
fdCOS (R)	5.4
paCOS	2.4
comCOS	3.3
$(GlcN)_2$	4.1
Trolox	0.05

3.7.2 Neuroprotective activity in SH-SY5Y neuroblastoma cells

The neuroprotective activity of fdCOS produced by Neutrase 0.8L towards human SH-S5Y5 neurons was tested. This sample was chosen because, although it was not the best in terms of antioxidant activity with ABTS, it was the easiest to produce, purify and characterize. First, viability of cells in the presence of fdCOS at three concentrations (0.02, 0.2 and 2 mg/mL) was assayed. fdCOS were not toxic for the cells (**Fig. 3.31A**), nor was DMSO. These concentrations were then tested for the neuroprotective activity in the presence of H_2O_2 (**Fig. 3.31B**). Values above 100% indicated neuroprotection. fdCOS showed a dose-dependent behavior increasing cells viability after exposure to hydrogen peroxide (**Fig. 3.31B**). In particular, the activity was higher at the lowest assayed concentration (0.02

mg/mL), at which the neuroprotective effect was statistically significant. The effect was slightly better than the observed with fructooligosaccharide standards.

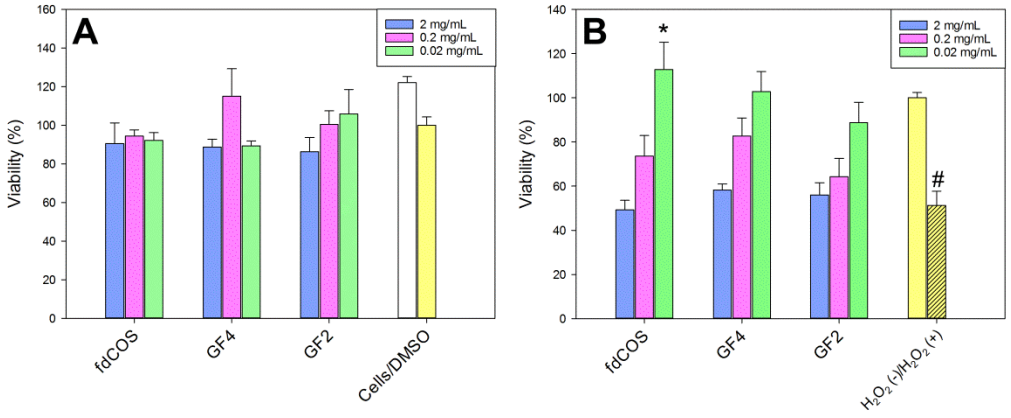


Fig. 3.31. *In vitro* analysis of neuroprotective activity of fdCOS. (**A**) Cell viability assays on SH-SY5Y neuronal cells. (**B**) Neuroprotective activity. Abbreviations: GF4: 1^{F}-fructofuranosyl-nystose; GF2: 1-kestose; H_2O_2 (-): Viability in presence of DMSO; H_2O_2 (+): Viability in presence of DMSO + H_2O_2. The data is expressed as mean ± SE (n=8, $^{*}p < 0.05$ *vs.* H_2O_2 (+) group; $^{\#}p < 0.01$ vs. H_2O_2 (-) group).

4 GENERAL DISCUSSION

4.1 FOS production by levansucrase from *Z. mobilis*

The effect of reaction conditions on the product profile of the levansucrase from *Z. mobilis* (LEV), as well as the immobilization of the enzyme with different strategies and the characterization of the immobilized biocatalysts was studied. LEV was first described by Lyness and Doelle in 1983 (Lyness and Doelle, 1983) and was previously studied in terms of production, purification and reaction products (Yanase et al., 1992, Bekers et al., 2002, Goldman et al., 2008).

In this Thesis we have analyzed in depth the composition of the reaction mixtures using different experimental conditions of sucrose concentration and temperature. **Fig. 4.1** summarizes all the different paths that sucrose can follow with levansucrase from *Z. mobilis* as biocatalyst. Once the fructosyl-enzyme intermediate is formed, different acceptors can participate in the process. The three major trisaccharides (1-kestose, neokestose and 6-kestose), which are formed when sucrose acts as acceptor, give rise to further elongation products. However, the homologous series of 6-kestose is the most determinant as it leads to the levan biopolymer. The levan may also contain β(2→1) branching points; the model compound of this group is the tetrasaccharide bifurcose (Velazquez-Hernandez et al., 2009). The released glucose can also act as acceptor yielding blastose, although this disaccharide can be also generated by hydrolysis of neokestose (Miranda-Molina et al., 2017). The fructose formed by the hydrolytic activity of levansucrase may also act as acceptor, producing fructosyl oligomers (levanbiose, levantriose, etc.) that have been also detected in reactions catalyzed by levansucrase (Tanaka et al., 1981).

We confirmed the dependency of the reaction conditions that was stated before (Vigants et al., 2013). Previously, the effect of ethanol and temperature was studied in order to obtain high yields of the polysaccharide levan. We went further by also analyzing the effect of the substrate concentration, which seems to be the key for the selectivity (transfructosylation or hydrolysis) of many glycosyl-transferases (Vega-Paulino and Zuñiga-Hansen, 2012). LEV showed higher T/H ratio at low temperatures, also displaying a higher selectivity towards the formation

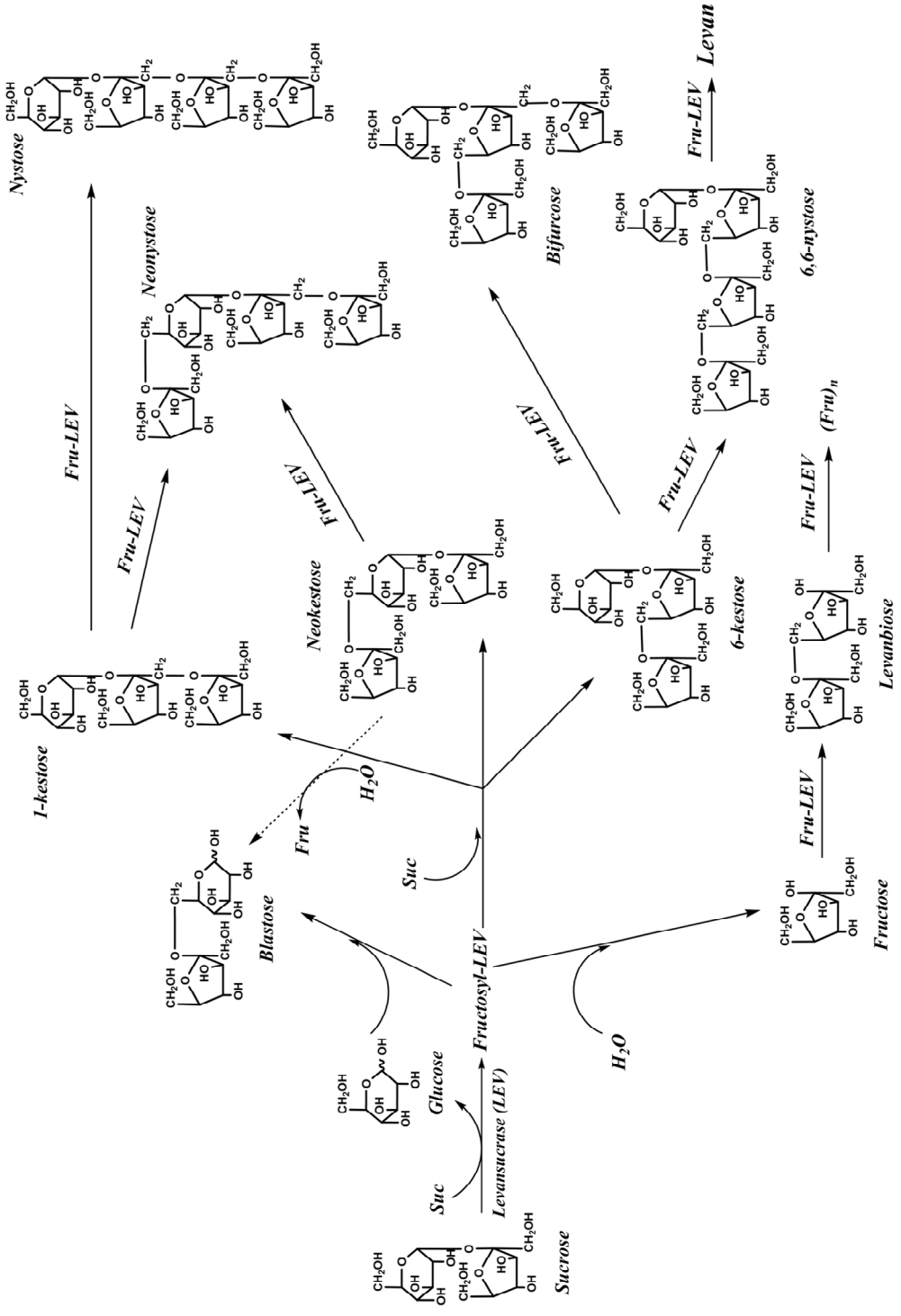


Fig. 4.1. Scheme of the formation of FOS and levan by levansucrase from *Z. mobilis*. Fructosyl-LEV and Fru-LEV refer to the fructosyl-enzyme intermediate.

of β(2→6) bonds and the polysaccharide levan. The T/H ratio was also higher with increasing sucrose concentrations, because the disaccharide competes with water as acceptor. When the temperature was 40ºC, at a high substrate concentration, LEV was less selective, forming FOS of different types, and only a small amount of the polysaccharide. Under the optimal conditions for the production of FOS, LEV yielded FOS of the levan family, but also inulin-FOS and neo-FOS. With so many different products, the identification of them all was challenging. More than 20 peaks were separated using HPAEC-PAD. With the aid of standards some FOS were identified: 6-kestose, 1-kestose, nystose, fructosyl-nystose and neokestose. Two more peaks were purified and identified by MS and NMR: blastose (Zambelli et al., 2014) –whose formation had already been described in the reactions with levansucrase from *B. subtilis*)- and 6,6-nystose (Santos-Moriano et al., 2015). This was a substantial improvement, both at the analytic level (Belghith et al., 2012, Borromei et al., 2009, Porras-Dominguez et al., 2014) and regarding the characterization of the levansucrase reaction (Bekers et al., 2002, Vigants et al., 2013, Homann et al., 2007, Li et al., 2011). However, some of the synthesized FOS could not be identified.

As explained in Chapter 1.2, FOS are of great interest due to their prebiotic properties. LEV showed a great activity and the ability to produce FOS of many types, whose synergistic effect could be of great significance. In order to produce FOS in a cost-effective way, LEV was immobilized. Two strategies were addressed: entrapment in desiccated calcium alginate (DALGEEs) and covalent immobilization. The first was selected because other fructosyl-transferases had worked quite well in DALGEEs (Fernandez-Arrojo et al., 2013). Unfortunately, immobilization of enzymes is very unpredictable, and in the case of LEV the apparent (experimental) activity of the biocatalyst and the recovery of activity were very low (**Table 4.1**). Our results indicated that we were able to entrap most of the enzyme, but the substrate was not able to reach the active site (**Fig. 4.2A** and **Fig. 4.2B**). Therefore, we decided to assess covalent immobilization, in which the enzyme is attached to certain groups of a carrier. Usually, the formation of covalent bonds takes place at alkaline pH, at which levansucrase is not stable (Results and Discussion, **Fig. 3.2**).

Fortunately, Ortega-Muñoz et al. had developed a vinyl sulfone-activated silica carrier that could bind proteins at neutral pH (Ortega-Munoz et al., 2010) and we used it successfully with LEV (Santos-Moriano et al., 2016a). From **Table 4.1** it is clear that, although the theoretical yield was very similar in both cases, the recovered activity was higher with the covalent immobilization. To obtain a biocatalyst with an activity close to 40 U/g, the covalent approach requires approximately 200-fold less enzyme units than the encapsulation strategy (**Table 4.1**). The presence of the enzyme on the carrier surface (**Fig. 4.2D**), and a smaller particle size (**Fig. 4.2A** and **Fig. 4.2C**) would explain the success of the covalent approach over the entrapment. To our knowledge, this was the first time that the levansucrase from *Z. mobilis* was covalently immobilized (Appendix I, **Table A4.1**).

Table 4.1. Comparison of the yields obtained with the different methods used for LEV immobilization.

		Theoretical[b]		Apparent[c]	
Biocatalyst	Initial[a] (U)	Activity (U/g)	Yield (%)	Activity (U/g)	Yield (%)
DALGEEs LEV	1900 ± 140	3500 ± 150	91 ± 10	42 ± 0.1	1.10 ± 0.07
DALGEEs LEV + TG	1800 ± 220	2800 ± 230	96 ± 18	38 ± 0.7	1.0 ± 0.13
LEV-VS (10:1 mg:g)	8.0 ± 0.5	152 ± 11	96 ± 13	40 ± 15	25 ± 9
LEV-VS (100:1 mg:g)	133 ± 0.4	2500 ± 100	93 ± 7	185 ± 5	7 ± 0.2

[a] Measured in a control of soluble levansucrase after incubation at the immobilization conditions.
[b] Theoretical values determined by subtracting the total initial activity prior to immobilization and the remaining activity in the filtrate and washing solutions after immobilization.
[c] Experimental activity (standard assay).

Table 4.2 summarizes every approach studied in this Thesis for the production of FOS with soluble and immobilized LEV. Production of FOS with both immobilized biocatalysts changed the selectivity of LEV towards a preferential formation of FOS of the inulin family, and the T/H slightly increased. These effects were probably due to small changes in the microenvironment of the enzyme. In the immobilizations of LEV from *Z. mobilis* described in the literature, all of them by non-covalent strategies (adsorption), formation of levan is reported in every case. This gives us an interesting hint about the effect that covalent binding can have on the selectivity of enzymes. In the case of other covalent immobilizations found in

the literature with levansucrases from other organisms (Appendix I, **Table A4.1**), mainly *Bacillus* sp., none of them shows a deep analysis of the reaction products. Instead, those publications are more focused on the immobilization yields and biocatalysts performance in general.

Selectivity of LEV reaction can be therefore modulated by changing the reaction conditions (temperature and sucrose concentration) and also by immobilizing the enzyme.

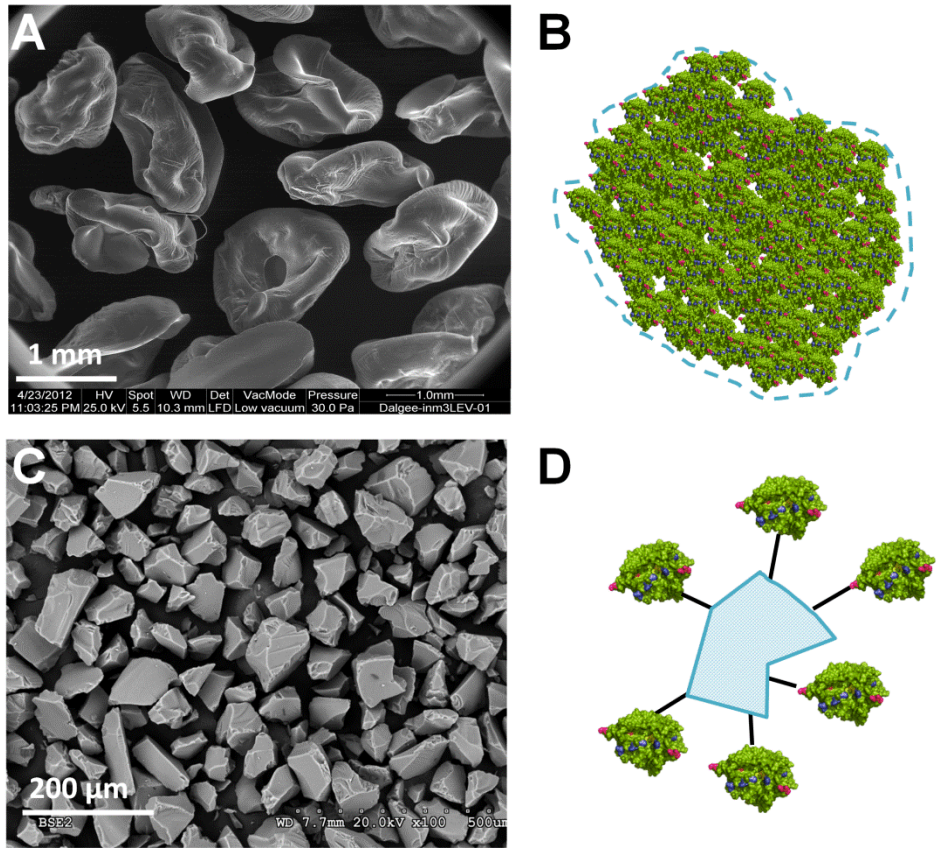
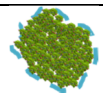


Fig. 4.2 . Methods of immobilization used with LEV. (**A**) DALGEEs of LEV visualized with SEM FEI QUANTA 200 at low vacuum conditions (30.0 Pa). (**B**) Schematic representation of the distribution of LEV molecules within the DALGEE. (**C**) LEV-VS visualized with SEM (100x). (**D**) Schematic representation of the distribution of LEV molecules on the surface of VS-activated silica. Figures B and C are not drawn to scale.

Table 4.2. Levansucrase reaction conditions to obtain different products.

Biocatalyst	T (°C)	Sucrose concentration (g/L)	T/H ratio	Main products
 Soluble LEV	4	100	3.8	HMW levan
		600	4.2	Small amount of FOS
	40	100	0.6	Fructose and glucose
		600	1.2	Inulin-FOS +++ Levan-FOS +++ Neo-FOS +++
 DALGEEs	40	600	1.8	Inulin-FOS +++ Levan-FOS ++ Neo-FOS ++
 LEV-VS	4	100	n.d.	n.d.
	40	600	1.7	Inulin-FOS +++ Levan-FOS + Neo-FOS +

+ low production; ++ moderate production; +++ high production.
n.d. not determined

4.2 Production of chitooligosaccharides

Six different enzymes were tested for the production of different chitooligosaccharides from chitosan. Three of them were commercial preparations with unspecific activity towards chitosan (BAN 480L, Neutrase 0.8L and Rapidase TF); one was a chitosanase extracted from a biopesticide preparation of *Bacillus thuringiensis* (Bt-chitosanase); and two of them were chitinases from *Tricchoderma reseii* expressed and produced in *Pichia pastoris* (CHIT33 and CHIT42).

Table 4.3 gathers the main differences between the six enzymes with chitosan as substrate. It illustrates how, although all of them are able to hydrolyze chitosan, the pattern of synthesized products is quite different. For instance, to produce only fully deacetylated COS (fdCOS), the best biocatalyst is Neutrase. On the contrary, for the production of only paCOS, a chitinase would be the enzyme of choice, because it requires at least one GlcNAc residue to cut the chitosan polymer.

Table 4.3. Differences in the pattern of chitooligosaccharides produced by the different enzymes studied in this Thesis with chitosan QS1 as substrate.

Enzyme	GlcN	GlcNAc	fdCOS	paCOS	faCOS
BAN 480L	✔	✘	✔	✔	✘
Neutrase 0.8L	✘	✘	✔	✘	✘
Rapidase TF	✔	✔	✔	✔	✘
Bt-chitosananse	✔	✘	✔	✔	✘
CHIT33	✘	✔	✘	✔	✔
CHIT42	✘	✔	✘	✔	✔

fdCOS: fully deacetylated COS; paCOS: partially acetylated COS; faCOS: fully acetylated COS.

The main difference between Bt-chitosanase and the commercial enzymes is its higher specific activity. Whilst in the case of Neutrase, BAN and Rapidase the protein concentration is very high and the activity is quite low, in the case of Bt-chitosanase it is the opposite.

Table 4.3 also demonstrates that the reaction mixtures are complex and difficult to analyze. In this Thesis, an analytic method was implemented using High Performance Anionic Exchange Chromatography with Pulsed Amperometric

Detection. This method represented an improvement compared with previous methodologies described in the literature, based mainly on HILIC and TLC analyses (Jiang et al., 2014, Li et al., 2012). There were also some previous reports on HPAEC-PAD analysis of chitin and chitosan oligosaccharides but using columns (PA1 or MA1) selective for small sugars (Xiong et al., 2009, Horsch et al., 1996, Lü et al., 2009) instead of the PA200 employed in this Thesis, more appropriated for oligosaccharides. In most cases the mobile phase was a diluted NaOH solution (15-25 mM) because the chitosan oligosaccharides are less tightly bound to the stationary phase than other carbohydrates, and thus require mild elution conditions to separate the different compounds; however, the resolution was generally poor and in some cases the analyses were too long (up to 3 h) (Horsch et al., 1996). Recently, van Munster and cols. (van Munster et al., 2015) improved the HPAEC-PAD separation of chitin oligosaccharides on a PA1 column using water as mobile phase followed by post-column treatment with NaOH to increase the sensitivity of the detector; the method was very specific for faCOS.

We have demonstrated that a CarboPack PA200 column was appropriate for the separation of complex mixtures of fdCOS and paCOS in less than 30 min employing a diluted NaOH solution (4 mM) as mobile phase (Materials and Methods, **Fig 2.1**). Our strategy was to compensate for the lower anion-exchange capacity of the PA200 column compared with the PA1 (35 µeq/column *vs.* 100 µeq/column) with the higher resolution of the PA200, provided a diluted NaOH mobile phase is used. This methodology represented a significant improvement compared with previous chromatographic protocols to separate COS. As the ionic strength of the eluent is below the specifications of the amperometric detectors, the sensitivity of the method (and thus the quantification of the products) could be further improved by implementing a post-column delivery system with concentrated NaOH (van Munster et al., 2015, Mekasha et al., 2016).

One of the commercial enzymes (BAN 480L) was covalently immobilized on glyoxal agarose beads. This preparation was chosen after a first immobilization screening with the three commercial enzymes. The immobilization and reactor set-

up was made in collaboration with the laboratory of Prof. John Woodley in the Technical University of Denmark, as part of a short stay.

Whereas there are several publications regarding the production of COS by soluble enzymes, there are fewer about immobilization (Appendix I, **Table A3.2**), and even less concerning reactors with immobilized chitosanolytic enzymes. Moreover, all of the publications showed productivities lower than those described in this Thesis (**Table 4.4**). As stated before in this Thesis, immobilization of enzymes is a great tool for the implementation of processes for the industrial production of bioactive carbohydrates. The main problem of the systems involving chitosan is the high viscosity of the substrate. We successfully developed a method based on a dual reactor with a first step in batch to reduce viscosity and a final step in continuous mode to produce high amounts of COS (Santos-Moriano et al., 2016b) (in bold in **Table 4.4**).

Table 4.4. Publications involving bioreactors for the production of COS with chitosan as substrate.

Immobilization method	Bioreactor	Yield[a] (%)	Productivity (g_{COS} $L_{REACTOR}^{-1}$ h^{-1})	Reference
–	Ultrafiltration membrane reactor *Batch*	44	2.2	(Sinha et al., 2014)
–	Ultrafiltration membrane reactor *Continuous*	75	9	(Kuo et al., 2004)
–	Ultrafiltration membrane reactor *Continuous*	55	13.2	(Lin et al., 2009)
Adsorption on chitin beads	Dual reactor: PBR + Membrane reactor *Continuous*	12.5[b]	0.19[b]	(Jeon and Kim, 2000)
Covalent attachment on agar gel	PBR *Continuous*	44	2.5	(Ichikawa et al., 2002)
Covalent attachment on agar-gel coated multidisk impeller	Stirred tank reactor *Batch*	22[c]	0.35[c]	(Ming et al., 2006)
Covalent attachment on agar gel	Ultrafiltration membrane reactor *Continuous*	46[c]	1.41[c]	(Kuroiwa et al., 2009)
Covalent attachment on agar gel	PBR *Continuous*	38[c]	1.46[c]	(Kuroiwa et al., 2003)
Covalent attachment to agarose beads	**PBR *Continuous***	**73**	**37**	**(Santos-Moriano et al., 2016b)**

[a]Proportion of chitosan hydrolyzed

[b]Referred to reducing sugars

[c]Referred only to the production of $(GlcN)_5$ + $(GlcN)_6$

4.3 Bioactivities of COS and FOS

COS possess antioxidant activity, as reported in previous studies (Chen et al., 2003, Eom et al., 2012, Li et al., 2013, Li et al., 2012, Ngo and Kim, 2014). This antioxidant capacity seems to be dose, DD and MW dependent (Je et al., 2004, Mengibar et al., 2013). In general terms, short COS with high DD are the most active. The mechanisms by which they exert this antioxidant capacity is not yet fully understood (Muanprasat and Chatsudthipong, 2017). Related with their antioxidant activity, COS might have an application in age-related diseases (Kerch, 2015).

There are some examples of COS with neuroprotective activity in the literature. For example, Xu and cols. suggested that COS might exert neuroprotective effect in rat cortical neurons against Cu^{2+}-induced cellular oxidative stress (Xu et al., 2010), as well as in glucose deprivation-induced cell apoptosis (Xu et al., 2011). Recently, Huang and cols. demonstrated the neuroprotective activity of COS with DP < 10 in a human neuronal cell line having potential application in therapies against Alzheimer's disease (Huang et al., 2015). The neuroprotective activity of five fdCOS was compared by Jiang et al. (Jiang et al., 2014) and they found that chitotriose induced the highest increase in Schwann cell survival. The mechanism of COS neuroprotection could be related with a decrease of intracellular reactive oxygen species (ROS), for example by complexing metal ions with the amino, hydroxyl and acetamide moieties present in COS. Recently, Joodi and cols. tried to elucidate the mechanisms by which COS exert this neuroprotective activity in PC12 cells and found that they modulate heat shock response proteins and MAPK phosphorylation (Joodi et al., 2011).

Antioxidant activity of COS can also be useful in the food industry, improving the shelf-life of products. For example, COS enhance beer flavour stability preventing formation of staling compounds and scavenging free radicals in staling beer (Yang et al., 2016). Also, chitosan has been used successfully as a preservative in meat, seafood and salmon (Knoor, 1986, No et al., 2007, Kim and Thomas, 2007). In these cases, chitosan seems to chelate the ferrous ion released from heme proteins in both meat and fish muscle, and therefore retarding lipid oxidation

(Tharanathan and Kittur, 2003). It is worth noting that in every case the best results were obtained with the chitosans with the lowest MW, suggesting that probably COS could exert a better effect. The antioxidant activity and natural origin, combined with their also proved antibacterial activity (Zhao et al., 2016), make COS very attractive biological preservatives.

In this Thesis two main families of COS were tested: fdCOS and paCOS, produced by combination of different enzymes and chitosans as substrate. We also tested some FOS mixtures, but none of them showed scavenging capacity on the ABTS radical.

The highest scavenging activity of ABTS radical was shown by paCOS. This was in agreement with previous studies by Li and cols. who demonstrated that a partially acetylated chitotriose showed higher antioxidant capacity than either the fully acetylated or deacetylated chitotrioses (Li et al., 2013). However, this mixture was quite difficult to obtain, with production yields under 2% and a high content in salts. That is why the mixture of fdCOS produced by Neutrase was chosen for its analysis in *in vitro* cell cultures. Commercial COS and COS produced by a combination of Rapidase and CHIT42 were also discarded due to the presence of the monomer GlcN. Glucosamine not only lacks any antioxidant activity, but it is also unspecifically cytotoxic (de Assis et al., 2012). fdCOS produced by Neutrase were well characterized and production yields of over 60% –after separation of the enzyme– were obtained. fdCOS showed neuroprotective activity in a statistically significant and dose dependent manner (Results and discussion, **Fig. 3.31**).

5 CONCLUSIONS

1. The temperature and substrate concentration strongly influence the product selectivity of levansucrase from *Zymomonas mobilis*: 4ºC and 100 g/L sucrose favors the formation of levan, whereas 40ºC and 600 g/L sucrose mainly yields inulin-type, levan-type and neo-type fructooligosaccharides (FOS).

2. Levansucrase was successfully immobilized at neutral pH on vinyl-sulfone activated silica with an apparent activity of 185 U/g. The immobilized biocatalyst was not able to synthesize levan, but produced similar amount of FOS than the free enzyme. It showed significant reuse stability in batch reactors.

3. It was possible to find out chitosanolytic activity in several commercial enzyme preparations: BAN 480L and Neutrase 0.8L (from *Bacillus amyloliquefaciens*), and Rapidase TF (from *Aspergillus niger*).

4. Neutrase produced only fully deacetylated chitooligosaccharides (fdCOS) without GlcN suggesting the presence of a deacetylating activity in the enzymatic preparation. We demonstrated that the protease was not responsible for the chitosanolytic activity, but it could probably catalyze the deacetylation process.

5. BAN produced a mixture of monomer-free fdCOS and paCOS with a yield close to 100% with different chitosans of varying DD and DP as substrate, suggesting that the enzyme responsible of the hydrolysis is an endochitosanase.

6. The chitosanolytic activity in BAN preparation was covalently immobilized on glyoxal agarose beads and the resulting biocatalyst showed great operational stability in batch. A dual reactor for the hydrolysis of chitosan was design whose productivity was higher than other bioreactors described in the literature.

7. Chitosanolytic activity was extracted from the biopesticide *Bacillus thuringiensis* (var. *aizawai*). The chitosanolytic activity isolated was of the endo-type yielding $(GlcN)_4$ as the main product.

8. The activity of two chitinases (CHIT33 and CHIT42) from *Trichoderma harzianum* was analysed with both chitin and chitosan as substrates. CHIT42 with chitosan as substrate produced exclusively paCOS, which were purified for the study of their biological properties.

9. By combination of HPAEC-PAD chromatography, mass spectrometry and bidimensional NMR it was possible to partially characterize the complex mixtures of FOS and COS obtained with levansucrase and chitosanases, respectively.

10. Some of the COS mixtures produced by the enzymes characterized in this Thesis displayed antioxidant activity. fdCOS produced by Neutrase with chitosan as substrate showed significant neuroprotective activity in human neuroblastoma cells.

1. La temperatura de reacción y la concentración de sustrato influencian de manera importante la selectividad de producto de la levansacarasa de *Zymomonas mobilis*. 4ºC y 100 g/L de sacarosa favorencen la formación levano, mientras que 40ºC y 600 g/L de sacarosa producen principalmente FOS de tipo levano, tipo inulina y neo-FOS.

2. La levansacarasa se inmovilizó de manera covalente a pH neutro en soportes de sílice vinil sulfona con una actividad aparente de aproximadamente 185 U/g. El biocatalizador inmovilizado no produjo levano, pero sí cantidades similares de FOS que la enzima soluble. LEV-VS demostró una gran estabilidad operacional en reactores *batch*.

3. Se encontró actividad quitosanolítica en tres preparaciones enzimáticas comerciales: BAN 480L, Neutrase 0.8L (ambas de *Bacillus amyloliquefaciens*) y Rapidase TF (de *Aspergillus niger*).

4. Neutrase produjo solo COS totalmente desacetilados sin GlcN sugiriendo la presencia de una actividad desacetilasa en la preparación enzimática. Se demostró que la proteasa no era responsable de la actividad quitosanolítica, pero que sí podría ser responsable de la actividad desacetilasa.

5. BAN produjo una mezcla de fdCOS y paCOS libre de monómeros con un rendimiento cercano a l00% con diferentes quitosanos de DD y DP variados como sustrato sugiriendo la presencia de una actividad endoquitosanasa.

6. La actividad quitosanolítica de la preparación BAN se inmovilizó covalentemente en soportes de glyoxal agarosa y el biocatalizador resultante mostró una gran estabilidad operacional en *batch*. Se diseñó un reactor dual para la hidrólisis de quitosano cuya productividad fue mayor que las de los biorreactores descritos en la literatura hasta la fecha.

7. Se extrajo actividad quitosanolítica del biopesticida *Bacillus thuringiensis* (var. *aizawai*). La actividad aislada era una endoquitosanasa que producía $(GlcN)_4$ como producto principal.

8. La actividad de dos quitinasas (CHIT33 y CHIT42) de *Trichoderma harzianum* producidas en *Pichia pastoris* se analizó con quitina y quitosano como sustrato. CHIT42 con quitosano como sustrato producía exclusivamente paCOS, que se purificaron para el análisis de sus propiedades biológicas.

9. Combinando técnicas de cromatografía (HPAEC-PAD), espectrometría de masas y RMN fue posible caracterizar parcialmente las complejas mezclas de FOS y COS obtenidas con levansacarasa y quitosanasas, respectivamente.

10. Alguna de las mezclas de COS producidas por las enzimas caracterizadas en esta Tesis mostraron actividad antioxidante. Los fdCOS producidos con por Neutrase con quitosano como sustrato mostraron actividad neuroprotectora significativa en células humanas de neuroblastoma.

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APPENDIX I

SUPPLEMENTARY MATERIAL

1. Protein gels

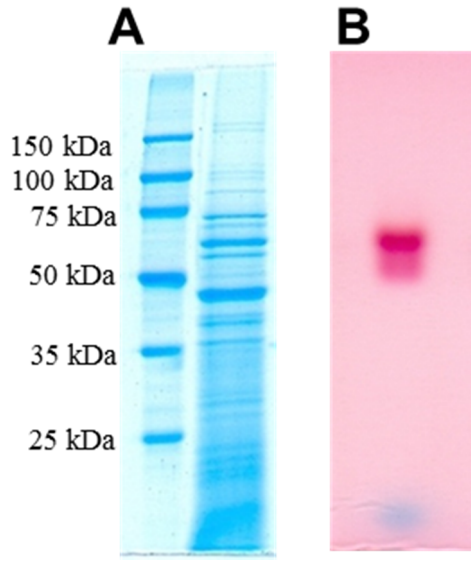


Fig. A1.1. Protein gels of levansucrase. (**A**) SDS-PAGE. Protein gel in denaturing conditions. Lane 1: MW markers. Lane 2: 0.1 mg/mL levansucrase. (**B**) ZYMOGRAM. Protein gel in native conditions showing the bands that produce reducing sugars.

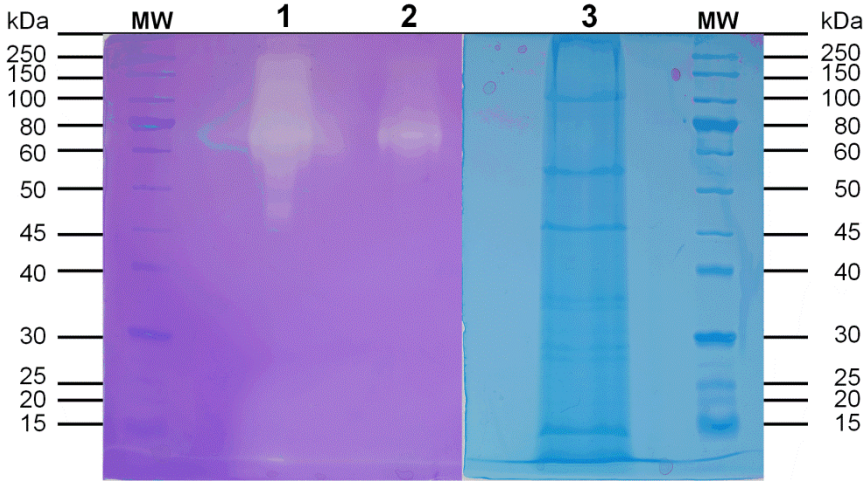


Fig. A1.2. Native polyacrylamide gel electrophoresis of the *B. thuringiensis* var. *aizawai* enzymatic extract (left, activity; right, protein). Lanes 1 and 2: extracts diluted 1:10 and 1:100, respectively; lane 3, extract not diluted. MW: molecular weight marker, sizes are indicated in kDa.

2. Mass spectra & NMR

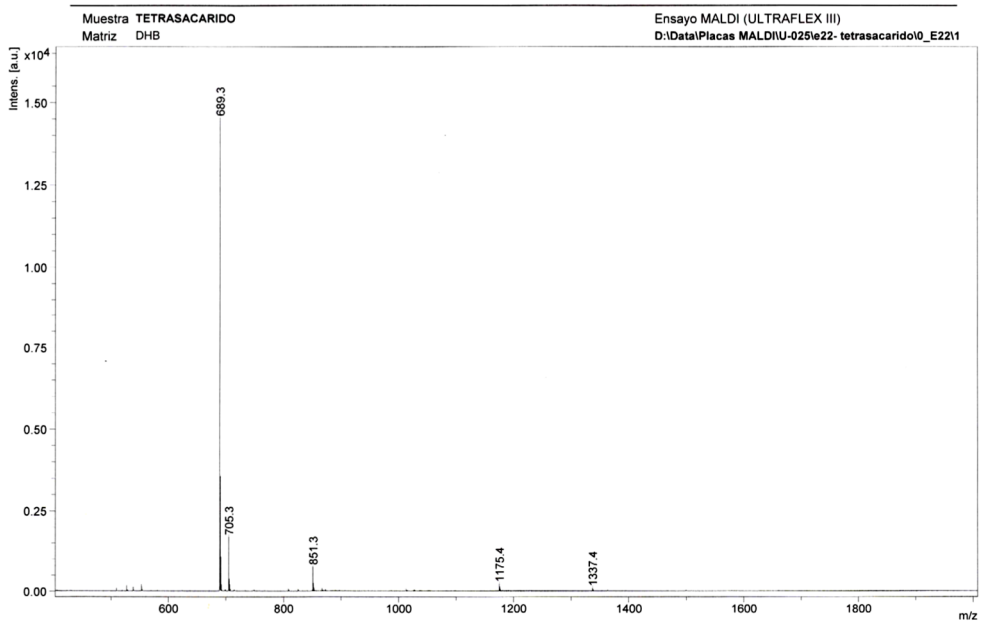


Fig. A2.1. MS-ESI of 6,6-nystose [Fru-β(2→6)-Fru-β(2→6)-Fru-β(2→1)-αGlc].

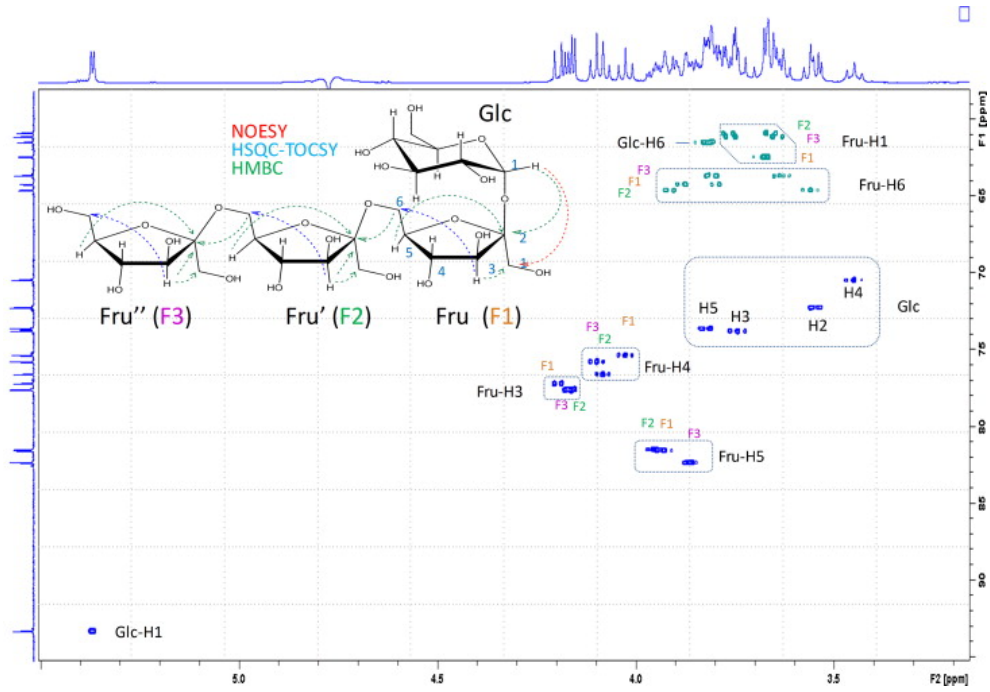


Fig. A2.2. 2D-NMR DEPT-HSQC spectra of 6,6-nystose [Fru-β(2→6)-Fru-β(2→6)-Fru-β(2→1)-αGlc]. The signals are assigned and labelled. The key points for identifications are also shown.

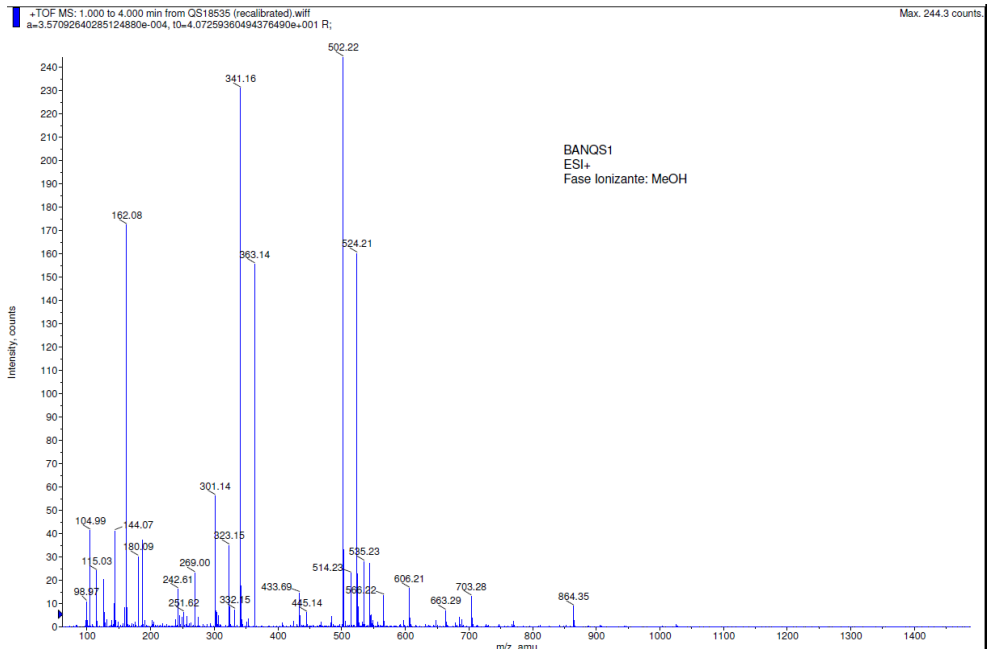


Fig. A2.3. MS-ESI of reaction mixture or BAN with chitosan QS1 as substrate.

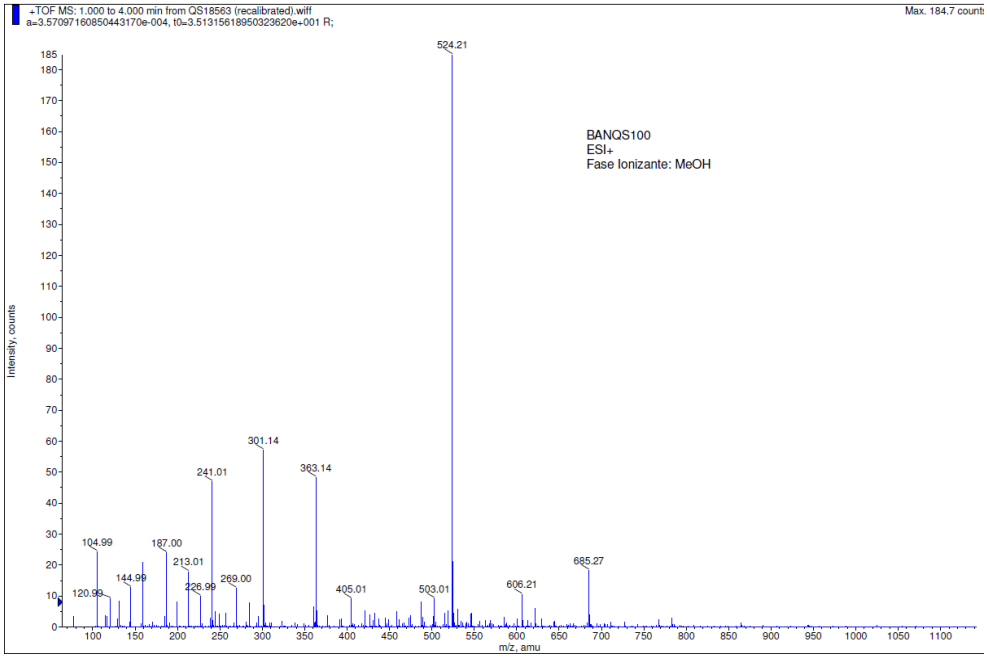


Fig. A2.4. MS-ESI of reaction mixture or BAN with chitosan CHIT100 as substrate.

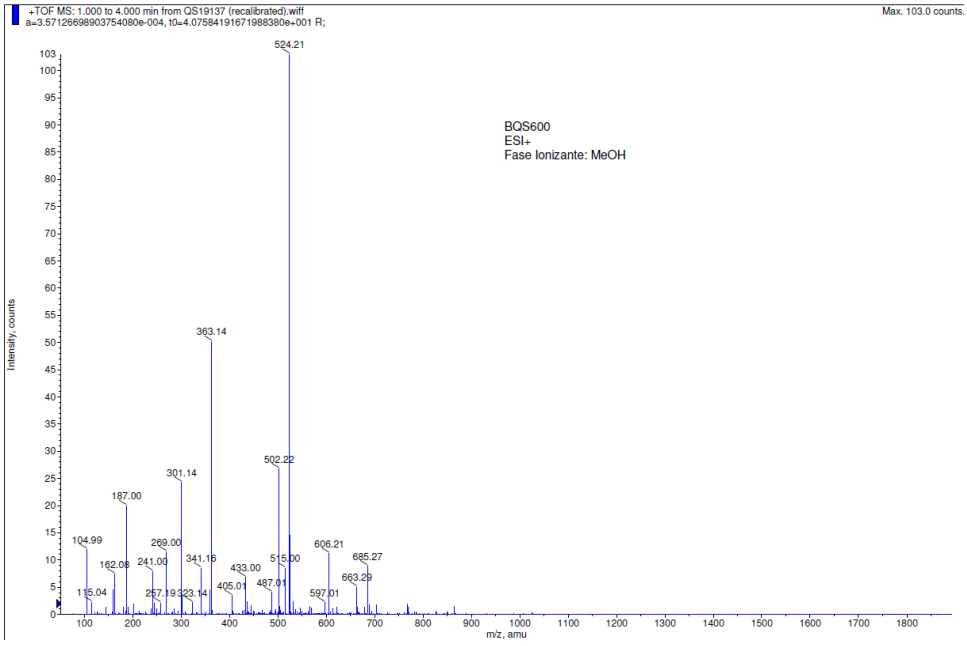


Fig. A2.5. MS-ESI of reaction mixture or BAN with chitosan CHIT600 as substrate.

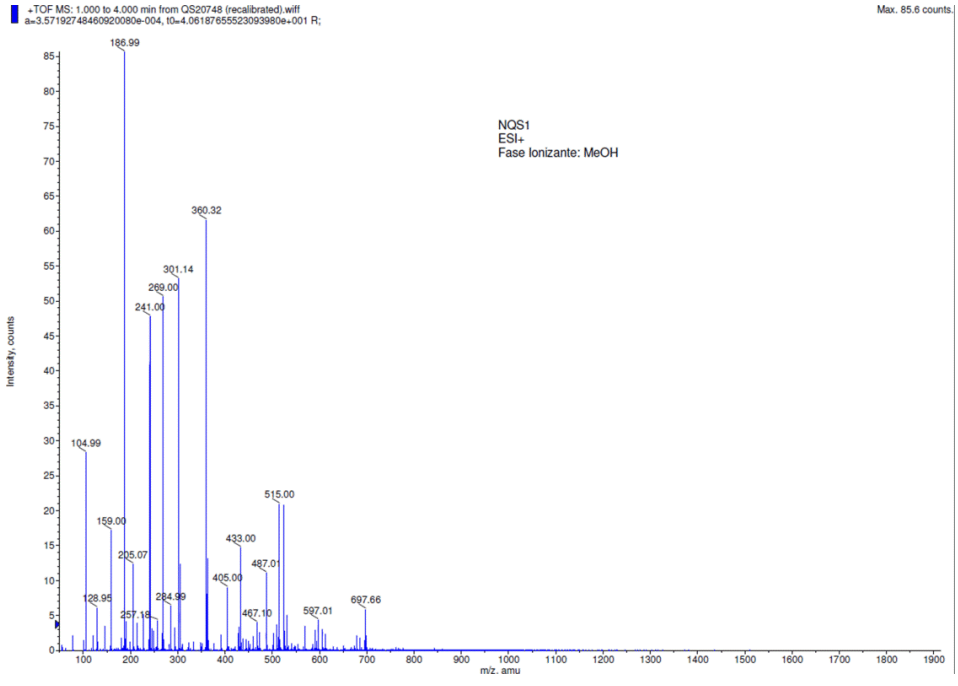


Fig. A2.6. MS-ESI of reaction mixture or Neutrase with chitosan QS1 as substrate.

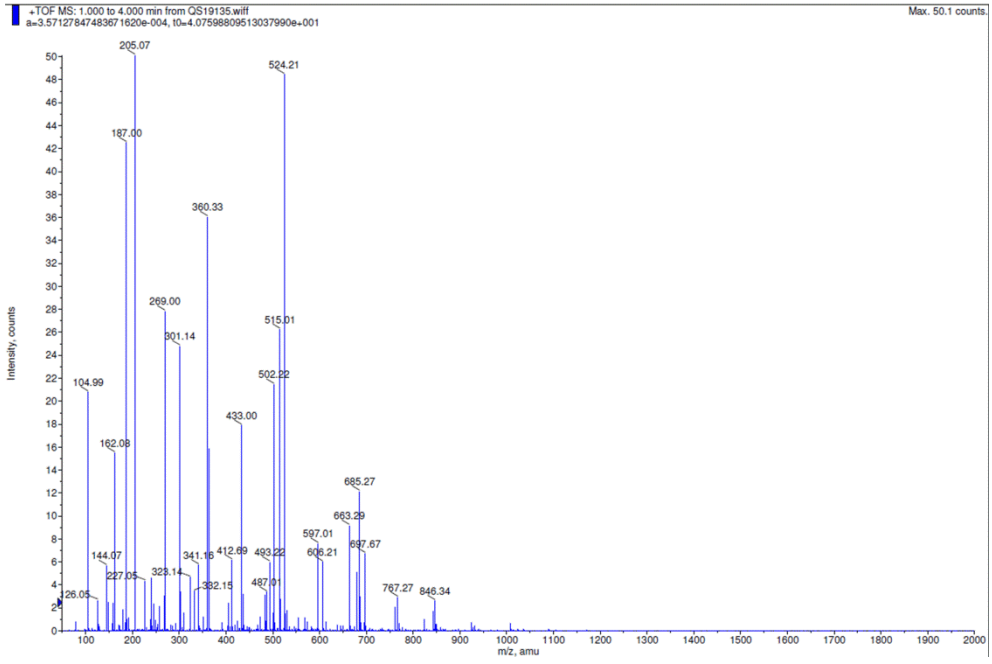


Fig. A2.7. MS-ESI of reaction mixture or Neutrase with chitosan CHIT100 as substrate.

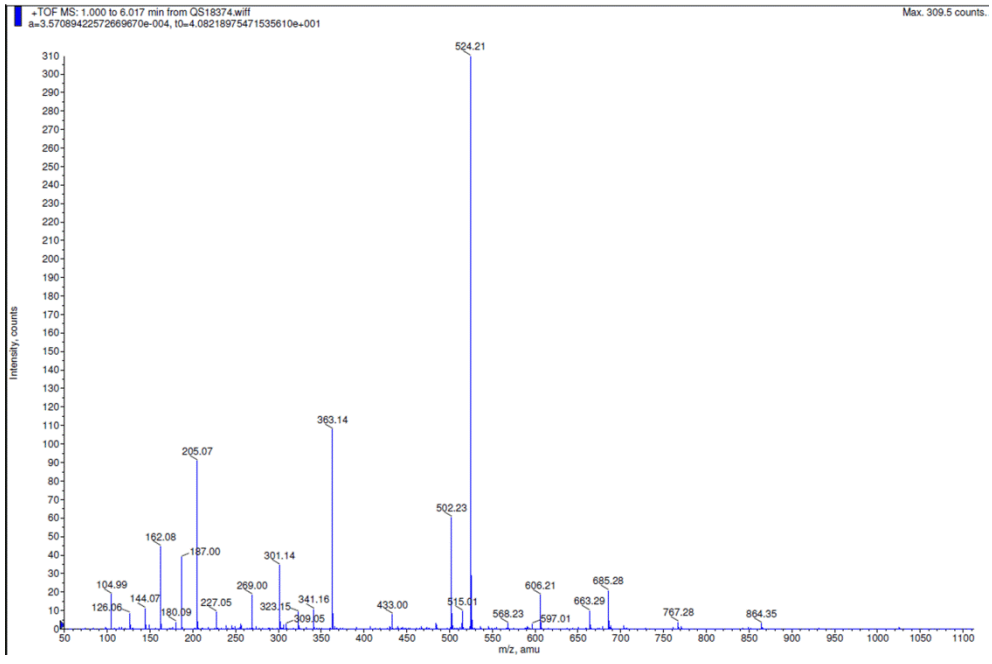


Fig. A2.8. MS-ESI of reaction mixture or Neutrase with chitosan CHIT600 as substrate.

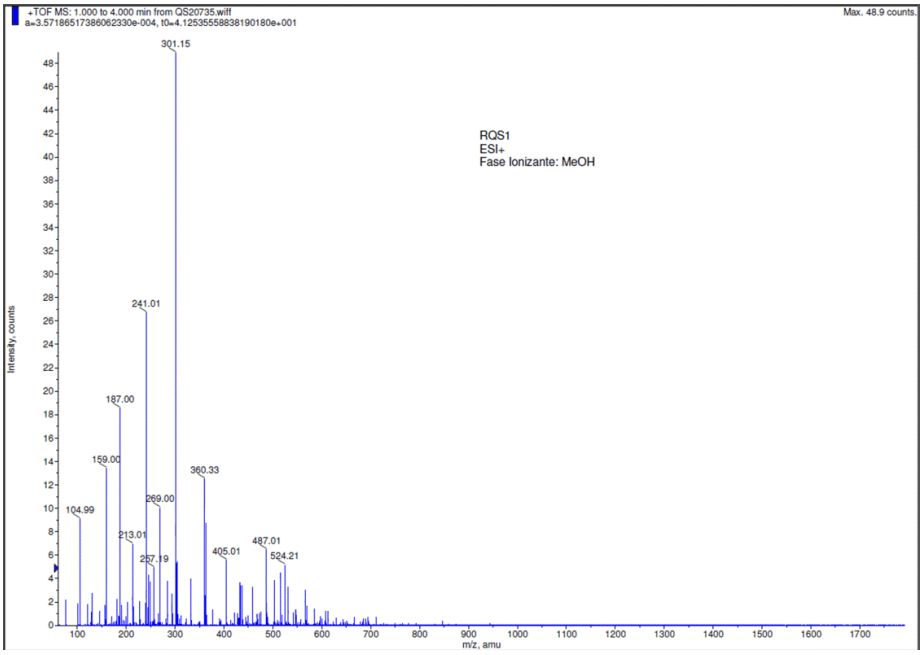


Fig. A2.9. MS-ESI of reaction mixture or Rapidase with chitosan QS1 as substrate.

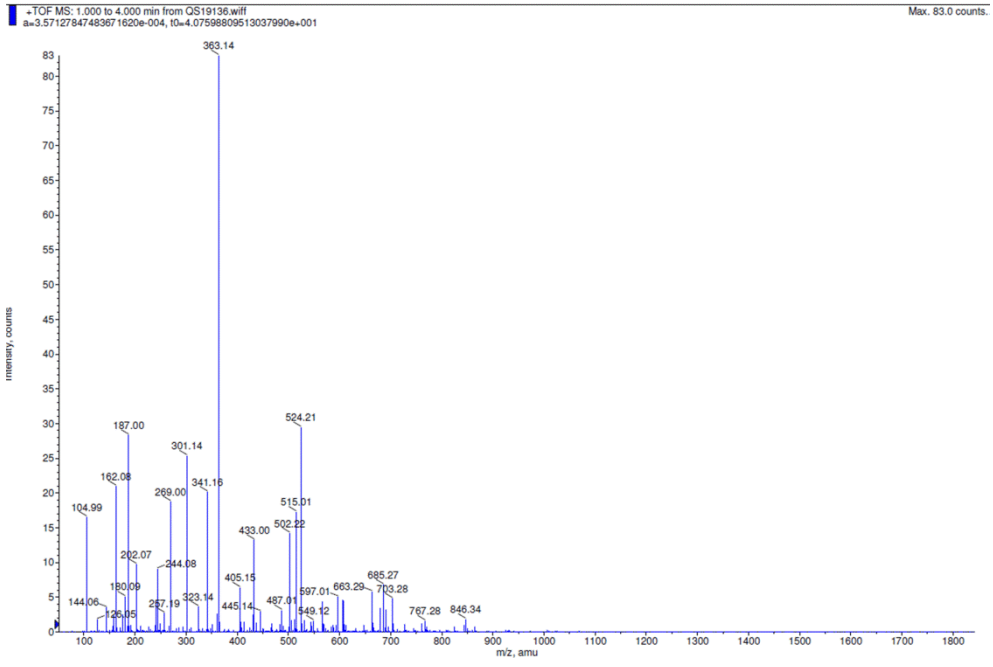


Fig. A2.10. MS-ESI of reaction mixture or Rapidase with chitosan CHIT100 as substrate.

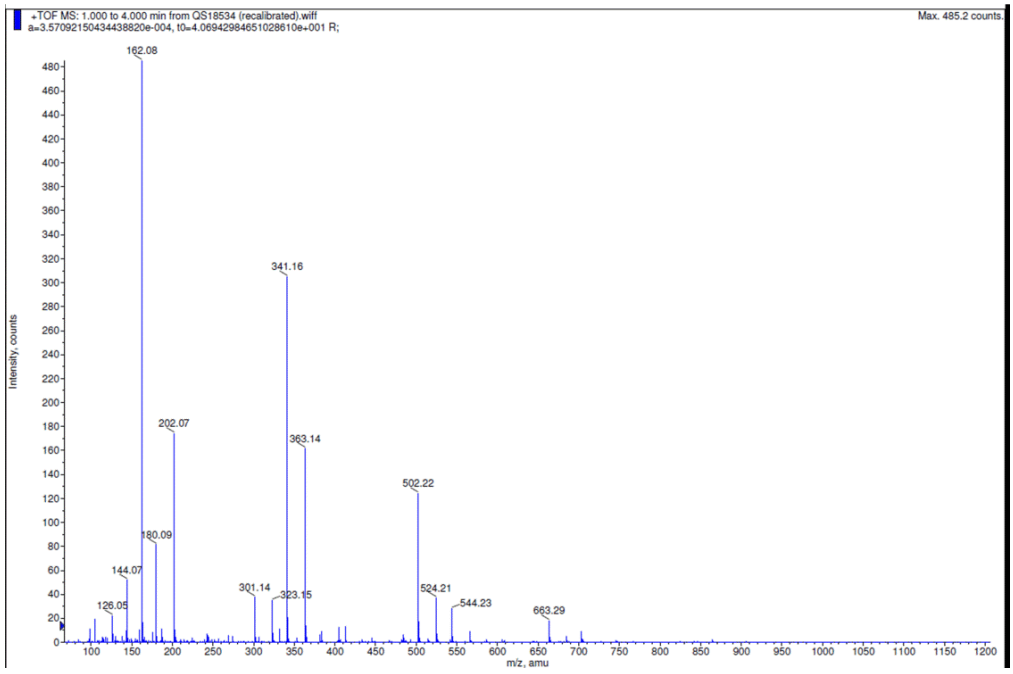


Fig. A2.11. MS-ESI of reaction mixture or Rapidase with chitosan CHIT600 as substrate.

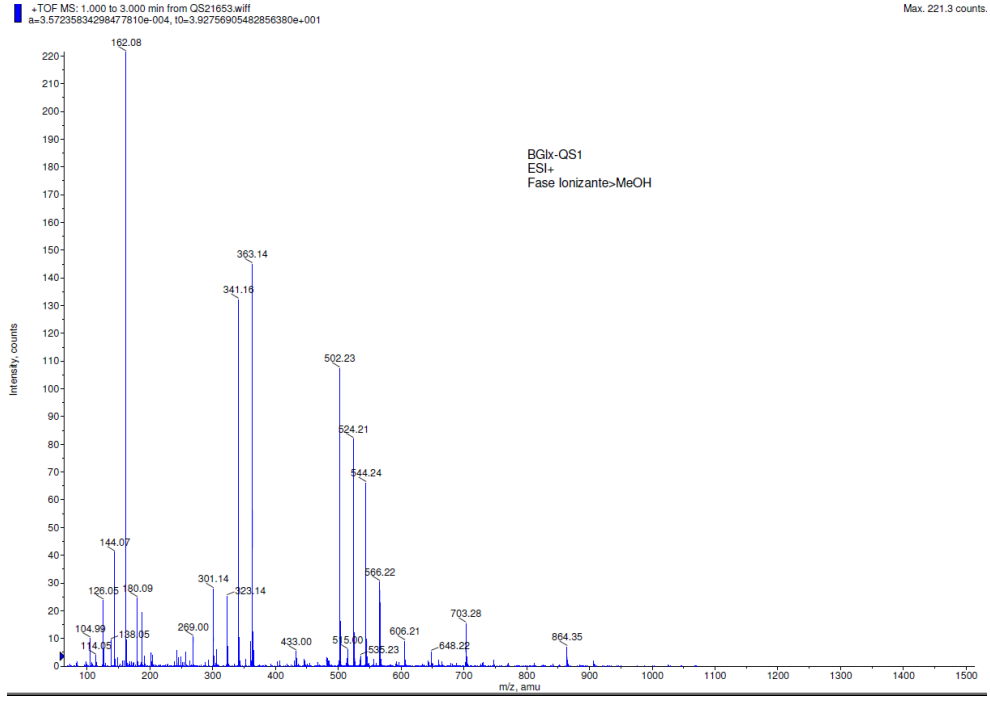


Fig. A2.12. MS-ESI of reaction mixture or BAN-Glx with chitosan QS1 as substrate.

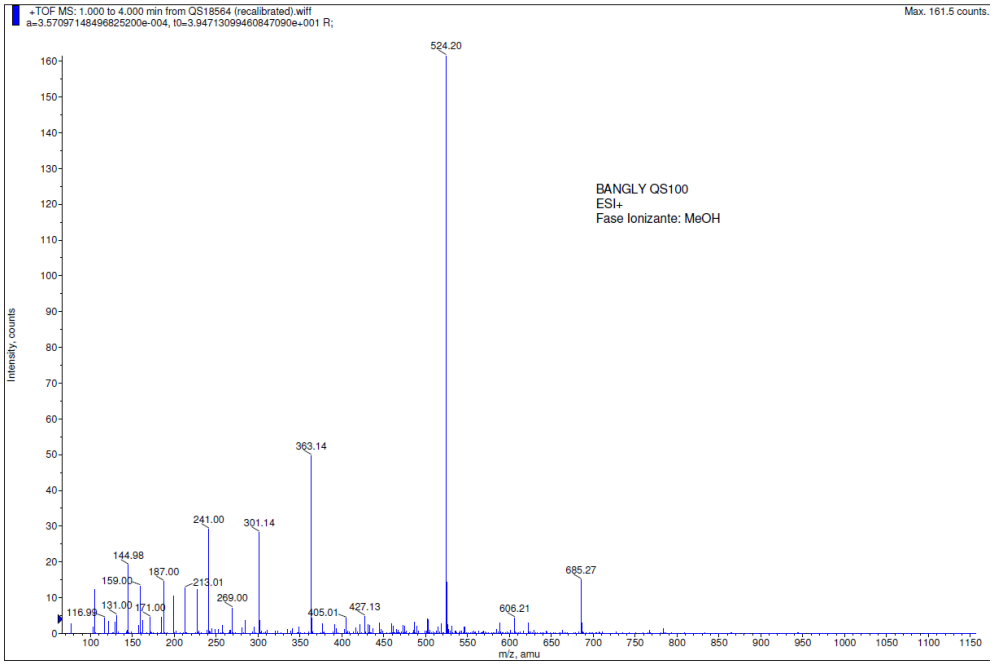


Fig. A2.13. MS-ESI of reaction mixture or BAN-Glx with chitosan CHIT100 as substrate.

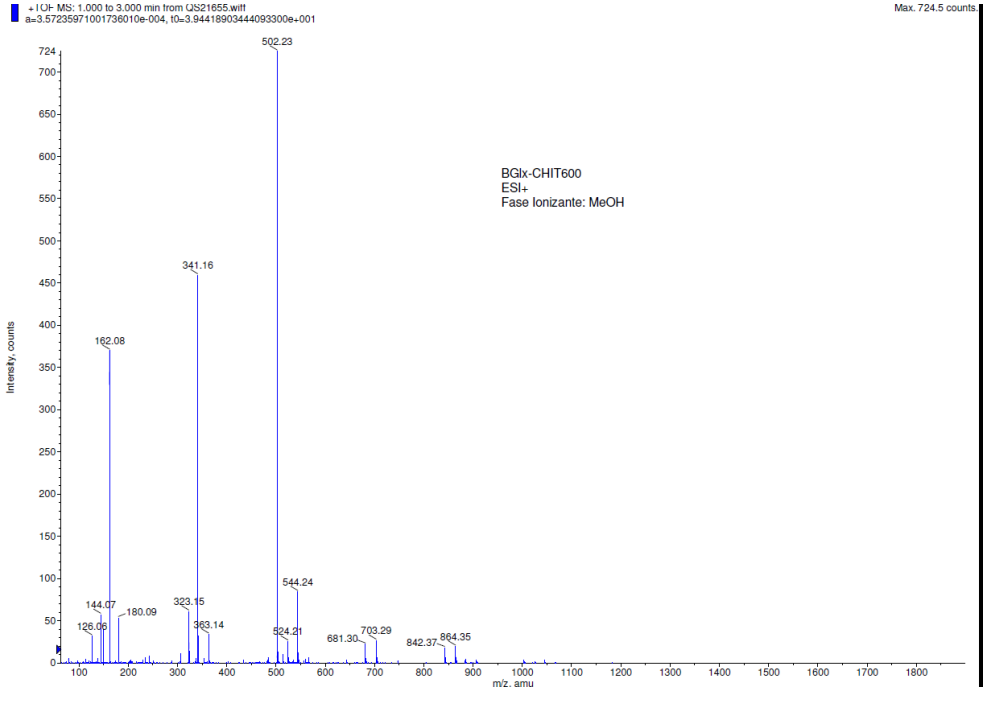


Fig. A2.14. MS-ESI of reaction mixture or BAN-Glx with chitosan CHIT600 as substrate.

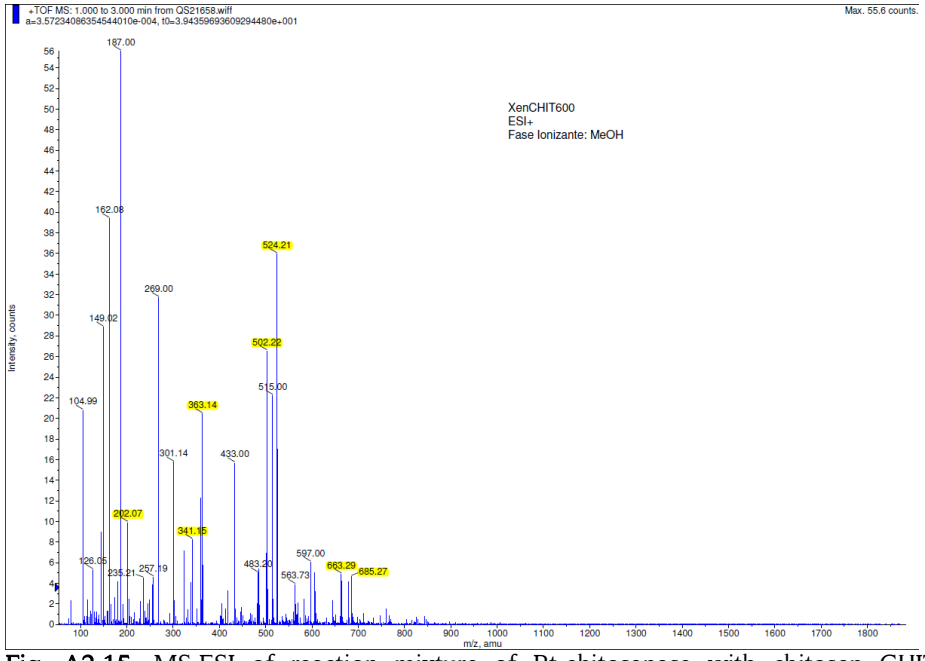


Fig. A2.15. MS-ESI of reaction mixture of Bt-chitosanase with chitosan CHIT600 as substrate.

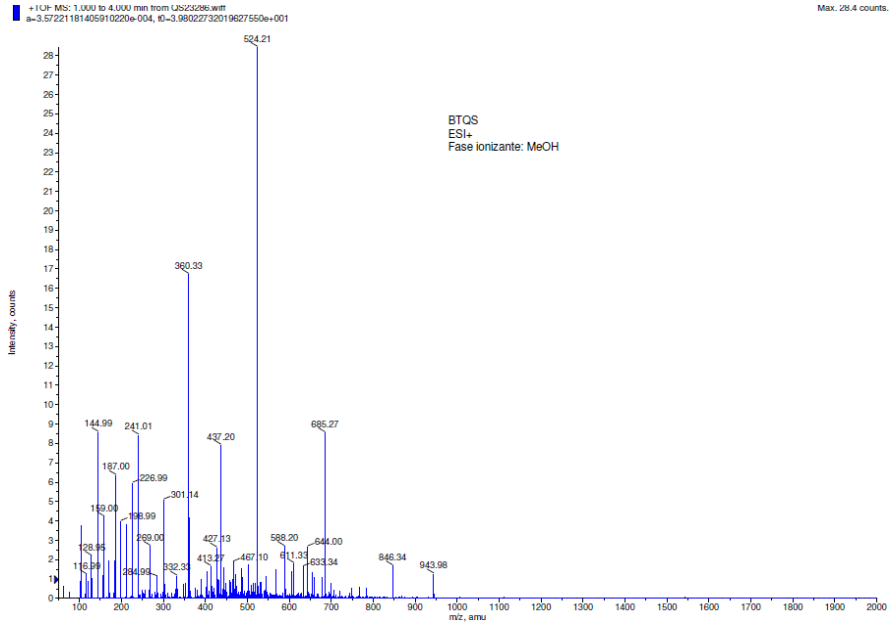


Fig. A2.16. MS-ESI of reaction mixture of Bt-chitosanase with chitosan QS1 as substrate.

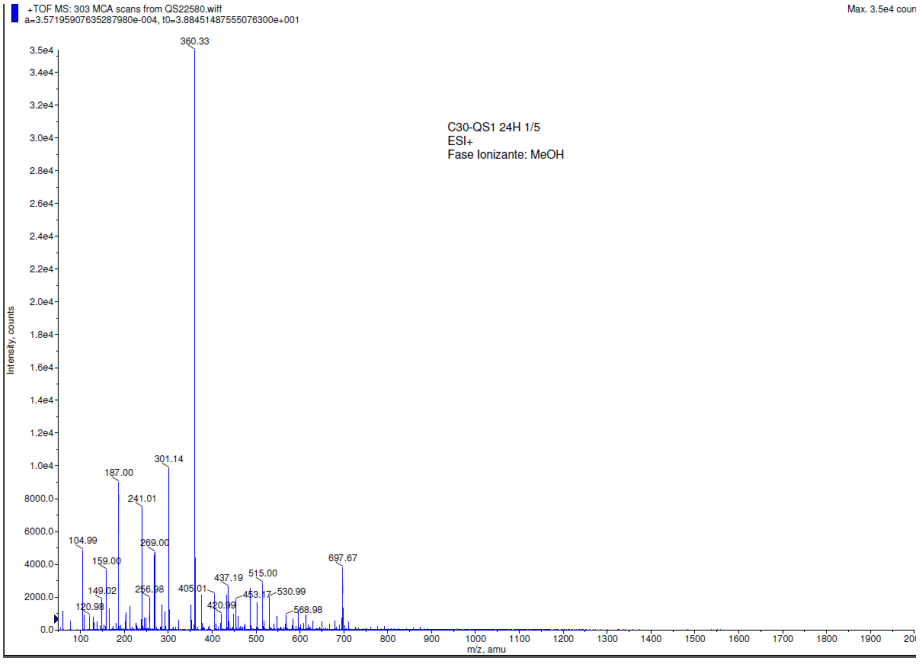


Fig. A2.17. MS-ESI of reaction mixture of CHIT33 with chitosan QS1 as substrate.

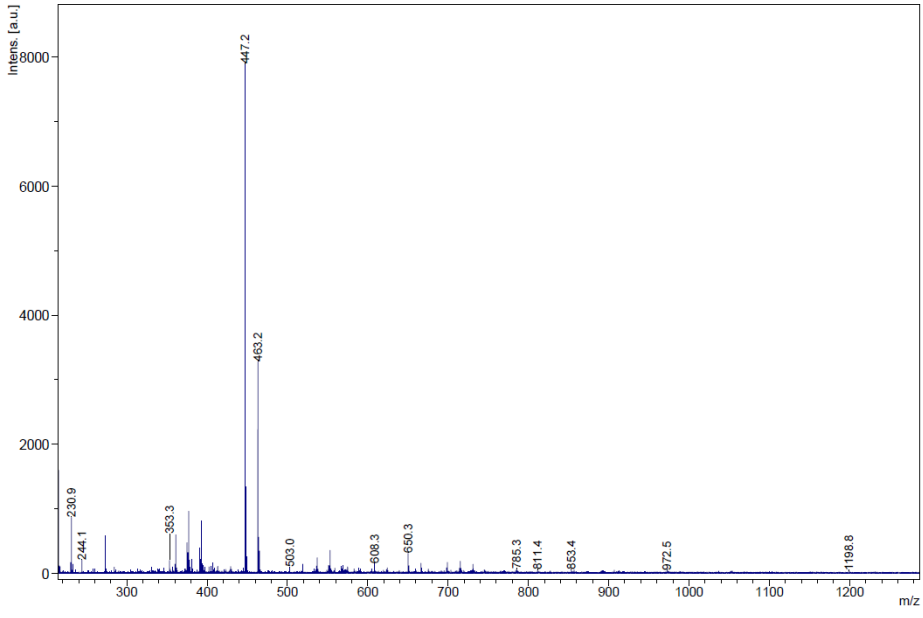


Fig. A2.18. MALDI-TOF of reaction mixture of CHIT42 with colloidal chitin as substrate.

3. Microscopy images

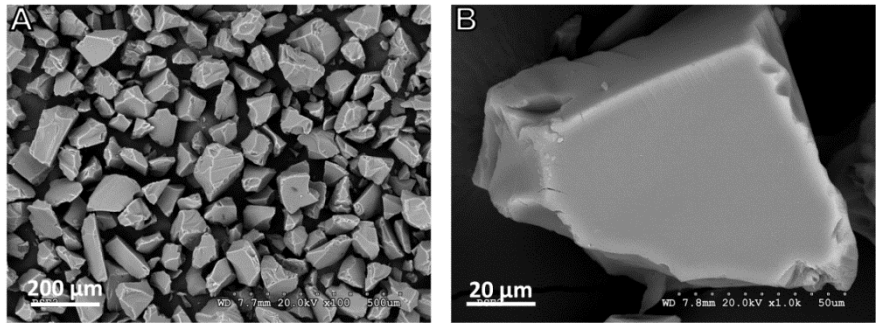


Fig. A3.1. Scanning Electron Microscopy (SEM) pictures of VS-activated silica: (**A**) 100x; (**B**) 1000x.

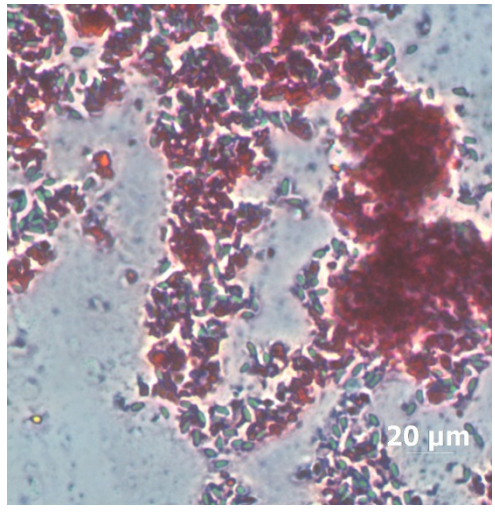


Fig. A3.2. Optical microscopy of *B. thuringiensis* cells and spores. Commercial preparation was dissolved in water and stained with malaquite green. Both alive cells (in red) and spores (in green) could be observed.

4. Tables

Table A4.1 Summary of publications involving immobilization of levansucrases.

Microorganism	Support	Type of immobilization	Reference
Bacillus amyloliquefaciens	Sepabeads HA Glyoxyl agarose	Covalent	(Hill et al., 2016)
Bacillus circulans	Dextran Wool	Covalent Physical adsorption	(El-Refai et al., 2009b) (El-Refai et al., 2009a)
Bacillus licheniformis	Chitosan Sepabeads EC-EP and HFA	Covalent	(Sangmanee et al., 2016)
Bacillus natto	Ceramic support SM-10	Physical adsorption	(Iizuka et al., 1993)
Bacillus subtilis	-	Cross-linking (CLEAs)	(Ortiz-Soto et al., 2009)
	Hydroxyapatite	Ionic adsorption	(Chambert and Petit-Glatron, 1993)
	Chitosan	Covalent binding through glutaraldehyde	(Esawy et al., 2008)
	Calcium alginate	Entrapment	
	Dowex 1-XB/DEAE-cellulose/Sephadex A-50	Ionic adsorption	
	Chitosan/ alumina/asbestos/ polyvinyl alcohol	Physical adsorption	
Zymomonas mobilis	Magnetite	Adsorption	(Jang et al., 2001)
	Oxidized stainless steel wire spheres	Adsorption	(Bekers et al., 1999)
	Chitin	Affinity binding	(Chiang et al., 2009)
	Hydroxyapatite	Ionic adsorption	(Jang et al., 2000)

Table A4.2. Summary of publications involving immobilization of chitosanolytic enzymes, including chitosanases, chitinases and other unspecific enzymes.

	Origin	Support	Type of immobilization	Reference
CHITOSANASES	*Bacillus pumilus* BN-262	Amylase-coated magnetic nanoparticles	Adsorption and multipoint covalent attachment	(Kuroiwa et al., 2008)
	Bacillus pumilus BN-262	Agar/Agarose gel	Multipoint covalent attachment with glicidol	(Kuroiwa et al., 2002)
	Bacillus pumilus BN-262	Agar gel	Multipoint covalent attachment	(Ichikawa et al., 2002)
	Bacillus pumilus BN-262	Agar gel-coated multidisk impeller	Multipoint covalent attachment	(Ming et al., 2006a, Ming et al., 2006b)
	Capsicum annuum	Chitin	Physical adsorption//covalent binding with glutaraldehyde crosslinking	(El-Sayed et al., 2016)
	Penicillium M2	CSG hybrid material	Covalent crosslinking	(Wang et al., 2012)
	Penicillium sp. ZDZ1	DEAE-cellulose	Covalent and glutaraldehyde crosslinking	(Zheng and Xiao, 2004)
	Penicillium sp. ZDZ1	Chitin	Adsorption and glutaraldehyde crosslinking	(Zeng and Zheng, 2002)
	Pseudomonas sp. CUY8	Sodium alginate/cellulose	Adsorption	(Wang et al., 2011)
	Streptomyces griseus	Silica gel	Glutaraldehyde crosslinking	(Song et al., 2014)
	Streptomyces griseus	Liposomes	Direct interaction	(Ngo et al.)
	Streptomyces zaomycetiucs C6	Electrospun PANNFM	Covalent	(Sinha et al., 2012)
CHTIINASES	*Thermomyces lanuginosus*	Chitosan	Covalent binding with glutaraldehyde crosslinking	(Prasad and Palanivelu, 2015)
	Thermomyces lanuginosus	Phenyl Sepharose	Hydrophobic adsorption	(Prasad and Palanivelu, 2014)
	Pseudomonas aeruginosa K-187	Hydroxypropyl methylcellulose acetate succinate	Adsorption	(Wang and Chio, 1998)
	Nocardia orientalis IFO 12806	Tannin-chitosan	Physical adsorption	(Sakai et al., 1991)
	Streptomyces griseus// Paenibacillus illinoisensis	Chitosan	Covalent binding with glutaraldehyde crosslinking	(Seo et al., 2012)

Table A4.2. (cont.) Summary of publications involving immobilization of chitosanolytic enzymes, including chitosanases, chitinases and other unspecific enzymes.

UNSPECIFIC ACTIVITY	*Aspergillus niger* "Pectinex Ultra"	Alginate	Adsorption	(Sardar et al., 2003)
	Carica papaya PAPAIN	Chitin	Covalent binding with glutaraldehyde crosslinking	(Lin et al., 2002)
	Cellulase	Chitin	Covalent binding with glutaraldehyde crosslinking	(Zheng et al., 2001)
	White egg lysozyme	Hydroxypropyl methylcellulose acetate succinate	Adsorption	(Chen et al., 2003)
	Rhodothermus obamensis "Branchzyme"	Agarose	Covalent binding with glutaraldehyde crosslinking	(Montilla et al., 2013)
	Trichoderma viride Cellulase	PAMAM-silica	Covalent binding	(Su et al., 2013)

5. Other Figures

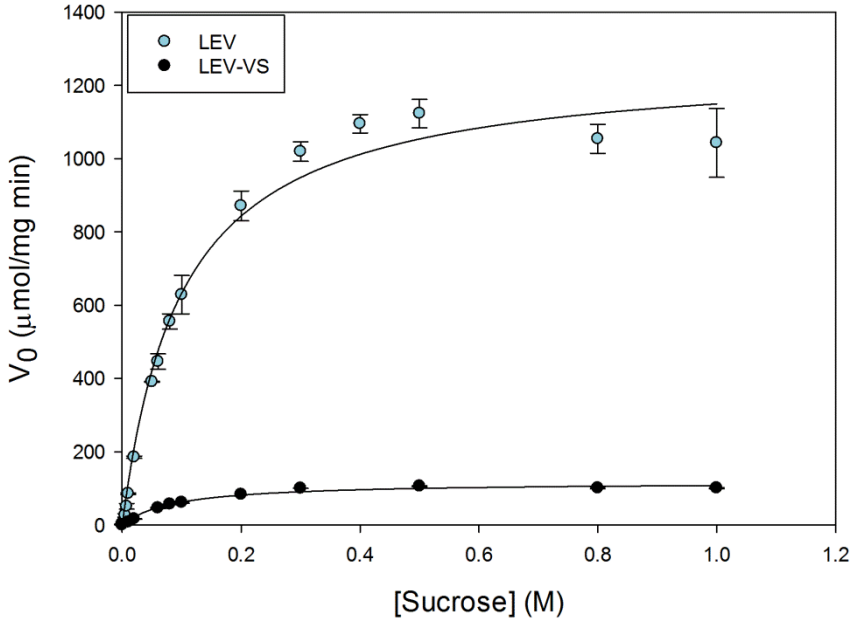
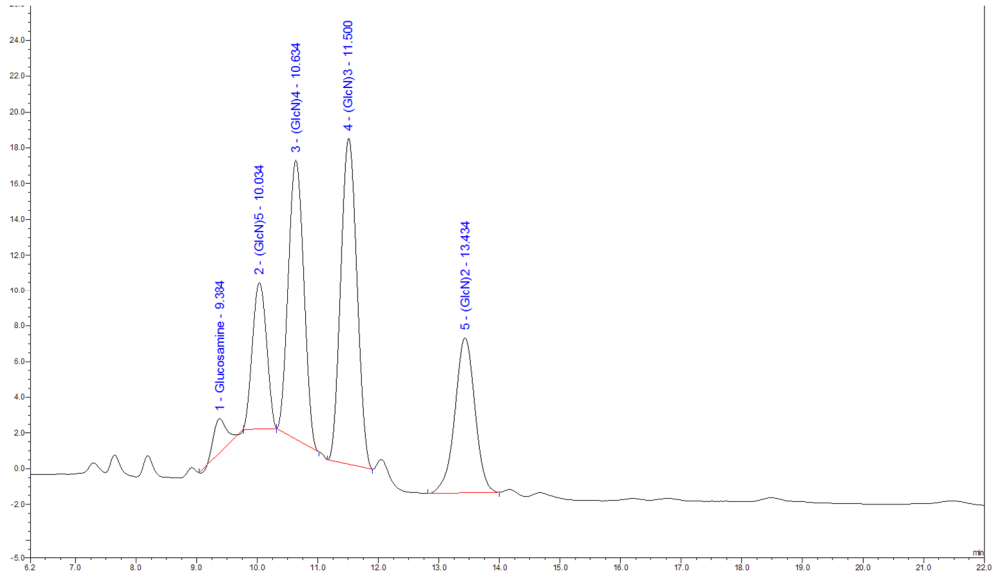


Fig. A5.1. Study of the kinetic parameters of levansucrase (LEV) and levansucrase immobilized on vinyl sulfone-activated silica carriers (LEV-VS).

A



B

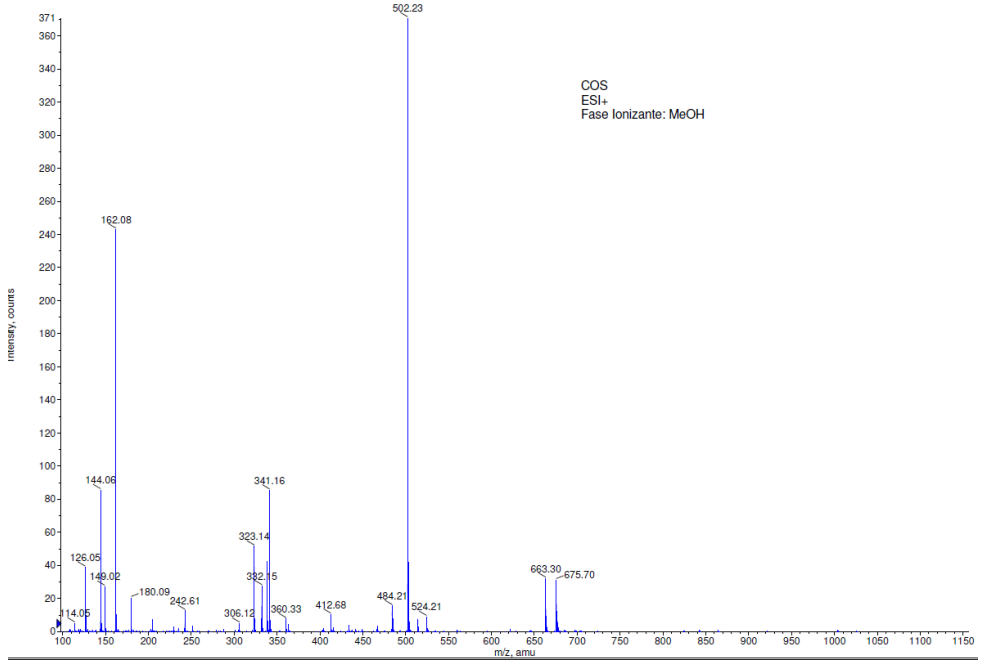
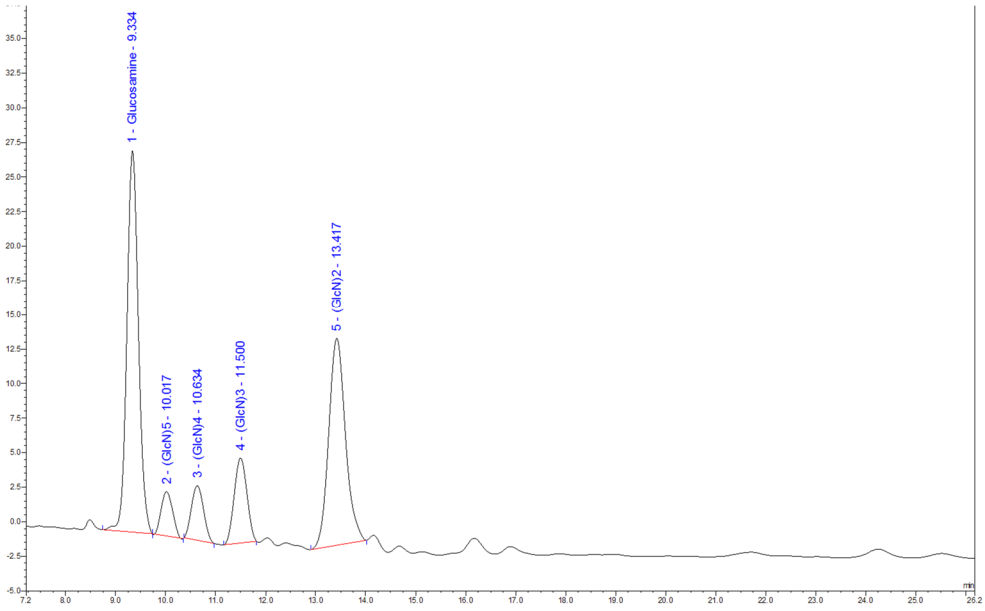


Fig. A5.2. Composition of fdCOS mixture produced by CHIT600 hydrolysis by Neutrase. (**A**) HPAEC-PAD and (**B**) MS-ESI.

A



B

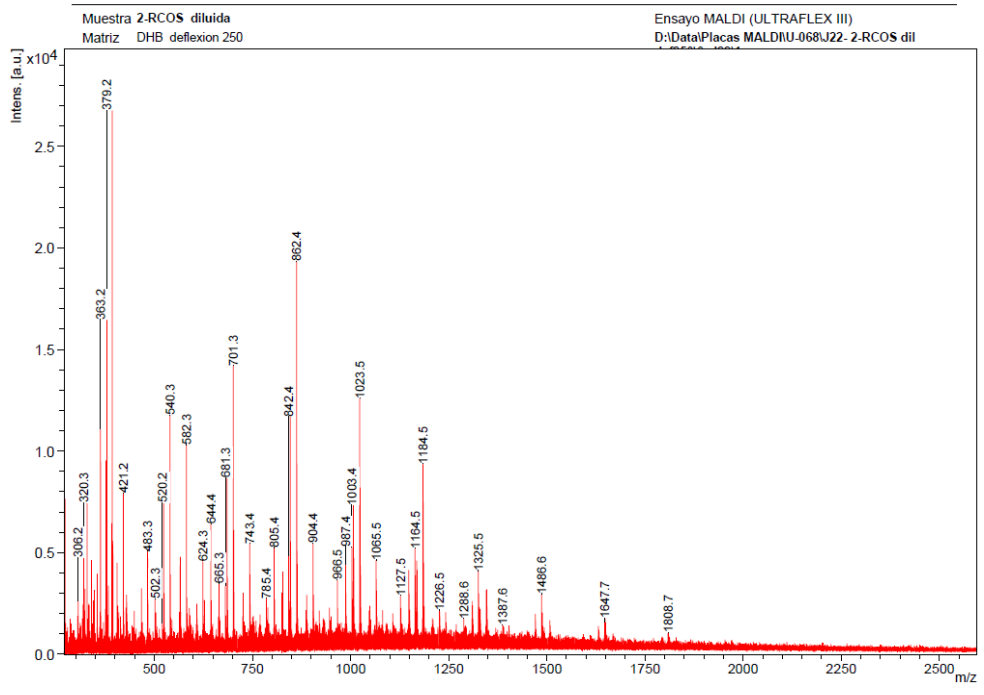
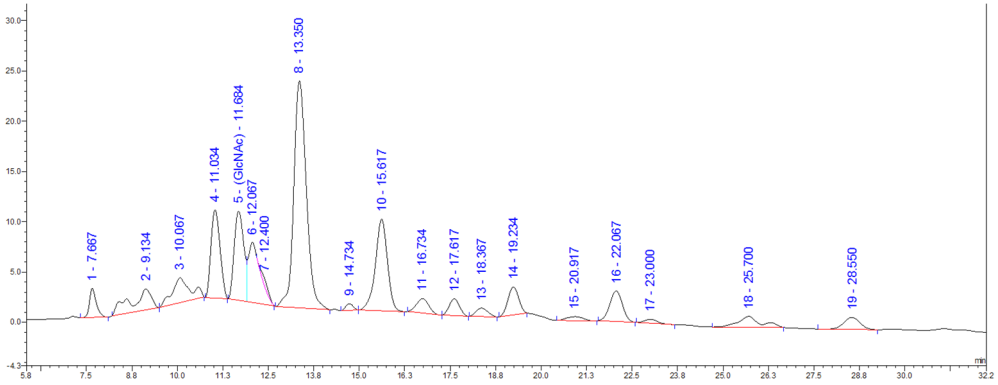


Fig. A5.3. Composition of fdCOS mixture produced by QS1 combined hydrolysis by CHIT42 and Rapidase. (**A**) HPAEC-PAD and (**B**) MS-MALDI.

A



B

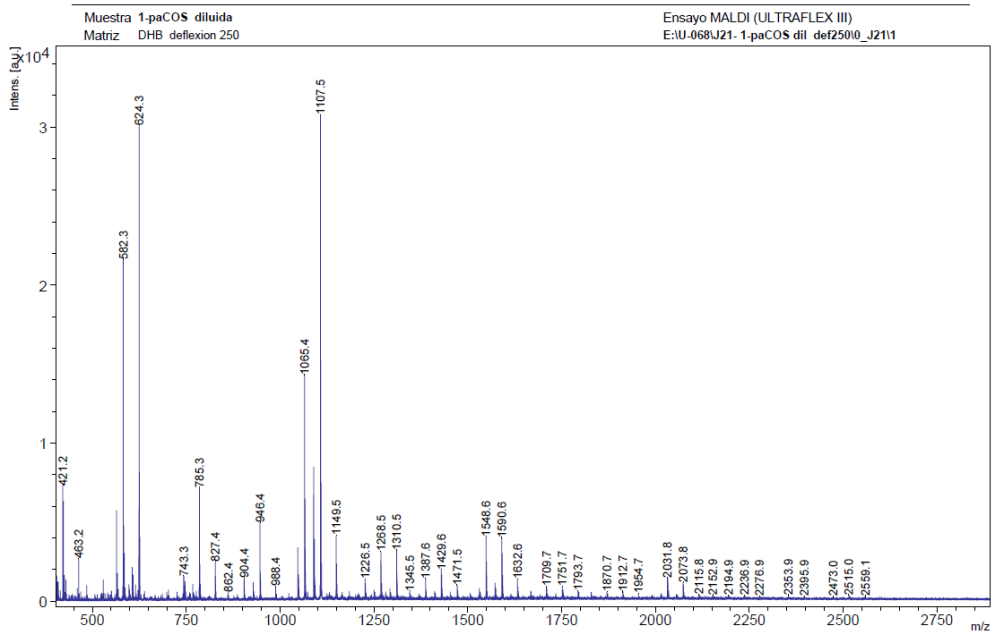
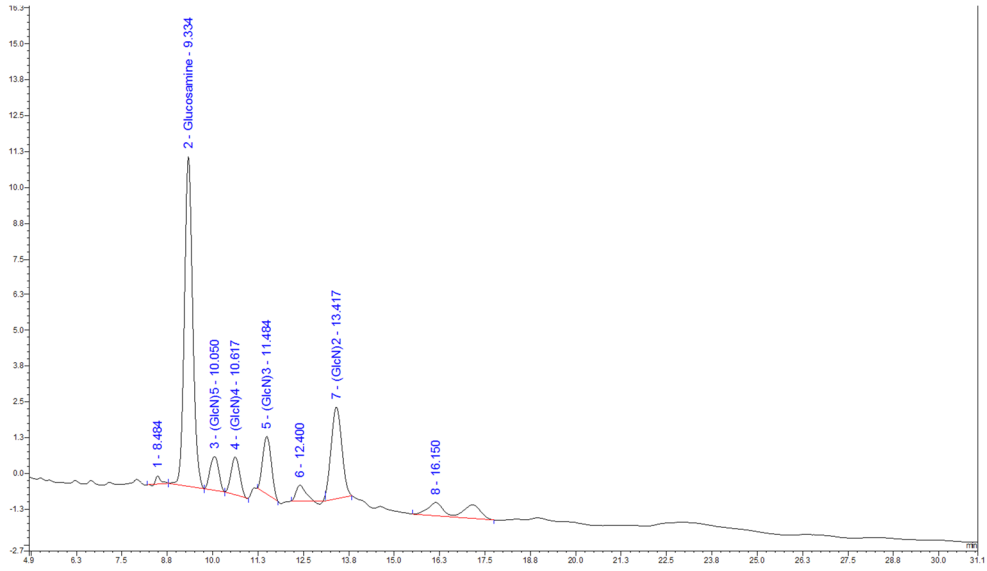


Fig. A5.4. Composition of paCOS mixture produced by QS1 hydrolysis by CHIT42. (**A**) HPAEC-PAD and (**B**) MS-MALDI.

A



B

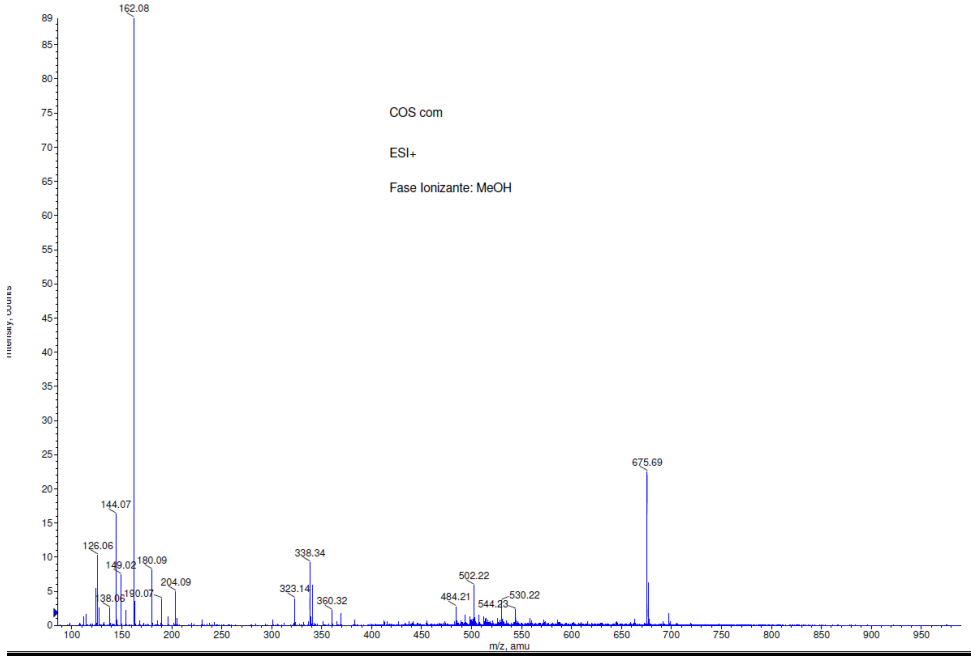
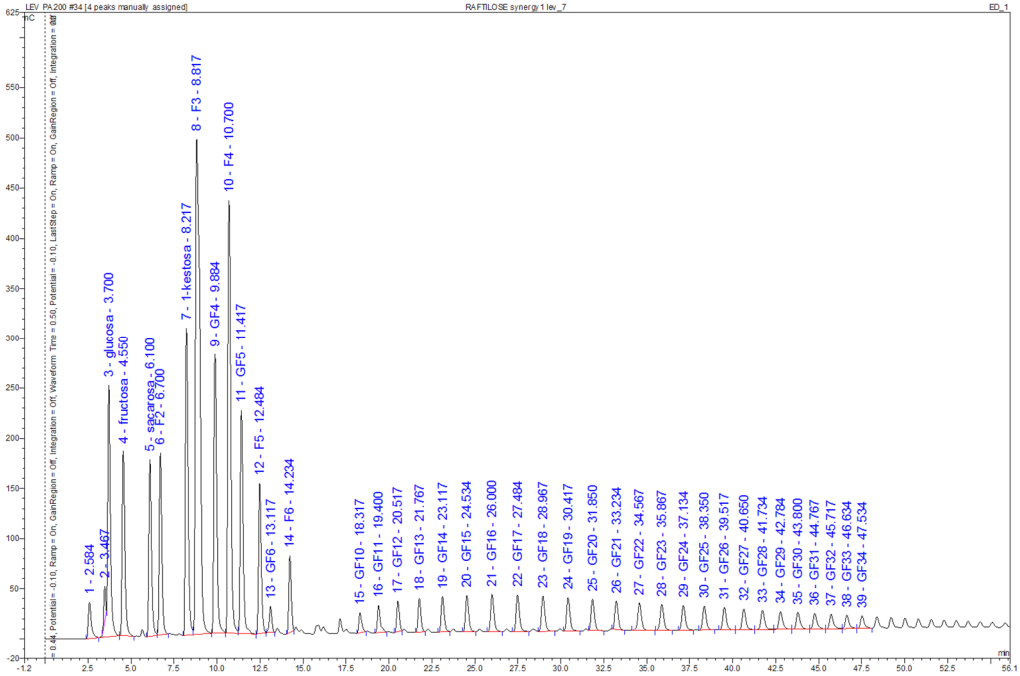


Fig. A5.5. Composition of commercial COS mixture (Qingdao BZ Oligo Biotech Co. Ltd. (China)). (**A**) HPAEC-PAD and (**B**) MS-ESI.

A



B

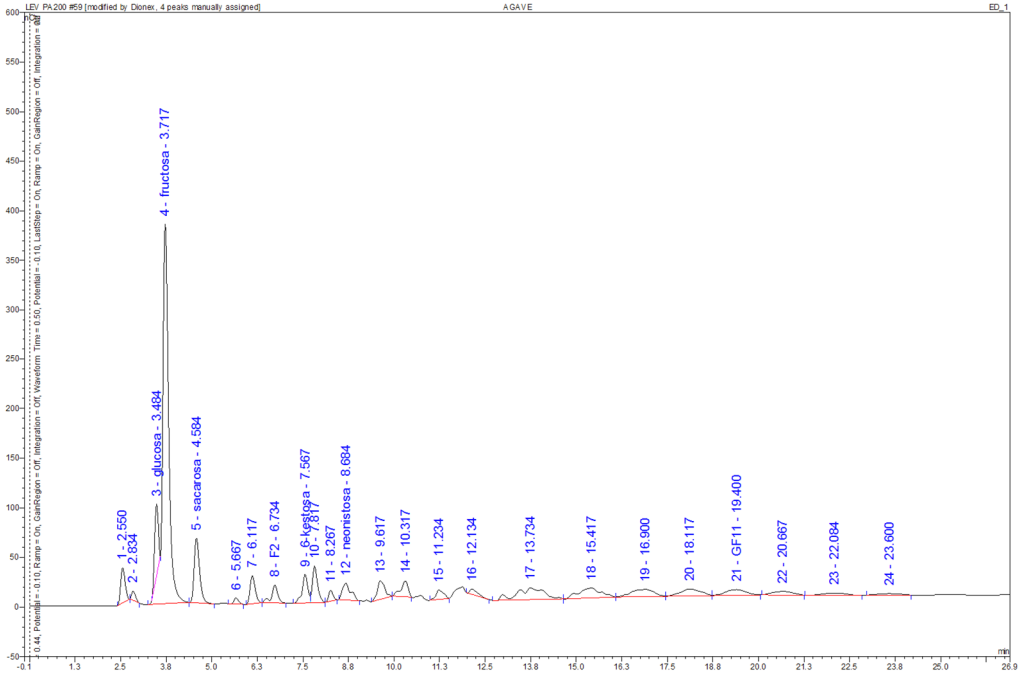


Fig. A5.6. Composition of FOS mixtures by HPAEC-PAD: (**A**) Raftilose (FOS produced by inulin hydrolysis) and (**B**) Agave (mixed FOS produced by hydrolysis of agave).

APPENDIX II

PAPERS

Publications related with the Thesis

Santos-Moriano, P., Fernandez-Arrojo, L., Mengibar, M., Belmonte-Reche, E., Peñalver, P., Acosta, N., Kidibule, P., Ballesteros, A.O., Morales, J.C., Fernandez-Lobato, M. & Plou, F.J. 2017. Enzymatic production of fully deacetylated chitooligosaccharides and their neuroprotective and anti-inflammatory properties. *Biocatalysis & Biotransformation, In press*, DOI: 10.1080/10242422.2017.1295231.

Santos-Moriano, P., Woodley, J.M. & Plou, F.J. 2016. Continuous production of chitooligosaccharides by an immobilized enzyme in a dual-reactor system. *Journal of Molecular Catalysis B: Enzymatic,* 133, 211-217.

Santos-Moriano, P., Monsalve-Ledesma, L., Ortega-Munoz, M., Fernandez-Arrojo, L., Ballesteros, A. O., Santoyo-Gonzalez, F. & Plou, F. 2016. Vinyl sulfone-activated silica for efficient covalent immobilization of alkaline unstable enzymes: application to levansucrase for fructooligosaccharides synthesis. *RSC Advances,* 6, 64175-64181.

Fernandez-Arrojo, L., Santos-Moriano, P., Rodriguez-Colinas, B., Ballesteros, A.O. & Plou, F.J. 2015. Micro-scale procedure for enzyme immobilization screening and operational stability assays. Biotechnology Letters, 37, 1593-1600.

Santos-Moriano, P., Fernandez-Arrojo, L., Rodriguez-Colinas, B., Ballesteros, A. & Plou, F.J. 2015. Enzymatic synthesis of fructooligosaccharides that stimulate the gut microbiota. *Anales de la Real Academia Nacional de Farmacia,* 81, 48-62.

Santos-Moriano, P., Fernandez-Arrojo, L., Poveda, A., Jimenez-Barbero, J., Ballesteros, A.O. & Plou, F.J. 2015. Levan versus fructooligosaccharide synthesis using the levansucrase from *Zymomonas mobilis*: Effect of reaction conditions. *Journal of Molecular Catalysis B: Enzymatic*, 119, 18-25.

Other publications

Rodriguez-Buey, M., Garcia-Ruiz, M., Martin-Diaz, J., Santos-Moriano, P., Molina-Espeja, P., Garcia-Ruiz, E., Plou, Francisco J., Alcalde, M. **2017**. Modified unspecific peroxygenase and uses thereof. Repsol S.A. & CSIC. European Patent P3816EP00 (27/03/2017)

Rodriguez-Colinas, B., Fernandez-Arrojo, L., Santos-Moriano, P., Ballesteros, A. & Plou, F. **2016**. Continuous Packed Bed Reactor with Immobilized β-Galactosidase for Production of Galactooligosaccharides (GOS). *Catalysts,* 6, 189.

Vicente, A. I., Viña-Gonzalez, J., Santos-Moriano, P., Marquez-Alvarez, C., Ballesteros, A. O. & Alcalde, M. **2016**. Evolved alkaline fungal laccase secreted by Saccharomyces cerevisiae as useful tool for the synthesis of C–N heteropolymeric dye. *Journal of Molecular Catalysis B: Enzymatic,* 134**,** 323-330.

Gimeno-Perez, M., Santos-Moriano, P., Fernandez-Arrojo, L., Poveda, A., Jimenez-Barbero, J., Ballesteros, A.O., Fernandez-Lobato, M. & Plou, F.J. **2014**. Regioselective synthesis of neo-erlose by the β-fructofuranosidase from *Xanthophyllomyces dendrorhous*. *Process Biochemistry,* 49, 423-429.

Plou, F.J., Fernandez-Arrojo, L., Santos-Moriano, P. & Ballesteros, A.O. **2014**. Application of immobilized enzymes for the synthesis of bioactive fructooligosaccharides. *Food Oligosaccharides: Production, Analysis and Bioactivity.*

Zambelli, P., Fernandez-Arrojo, L., Romano, D., Santos-Moriano, P., Gimeno-Perez, M., Poveda, A., Gandolfi, R., Fernandez-Lobato, M., Molinari, F. & Plou, F.J. **2014**. Production of fructooligosaccharides by mycelium-bound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicilium sizovae*. *Process Biochemistry,* 49, 2174-2180.